Food Research

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GROWTH OF FOOD SPOILAGE BACTERIA IN BANANA PURÉE

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Cambridge, Massachusetts

(Manuscript received April 29, 1959)

The antibacterial nature of a food indicates to some extent the minimum processing conditions for its preservation. A raw material with significant antibacterial quality may require milder heat processing and may retain more of its original, desirable qualities. Some factors that relate to bacterial inhibition are the pH *(4, 8, 13)*, the organic acid and sugar contents *(6, 10)* and the development of inhibitory substances during heating *(14)*. Other sources of antibacterial activity are inhibitory compounds peculiar to particular species of food plants. Florey *et al (3)* and Guyer and Erickson *(4)* have considered pH and organic acid factors. The possibility of peculiar antibacterial substances in bananas was indicated by Scott *et al (9)* and Dunn and Pettit *(2)*. Scott *et al (9)*, working with the methanolic extracts of airdried (85° C) banana pulp, noted significant antibacterial activity against nine species of molds and bacteria. They did not consider anaerobic spoilage organisms.

Dunn and Pettit *(2)* enlarged the antibacterial study with forty-two species of bacteria, yeasts, and molds, and considered the effects of banana ripeness, fresh vs dried and fresh vs heat-treated bananas, autoclaved banana extracts and ether extracts. In general, Dunn and Pettit encountered a low order of antibacterial activity against several of the varieties of bacteria, yeasts, and molds tested. *Escherichia coli*, *Serratia marcescens* and *Bacillus brevis* were partially inhibited, and *Torulopsis rosea* and *Endomycopsis fibuliger* were strongly inhibited by fresh banana pulp. Under anaerobic conditions, *Escherichia coli*, *Escherichia freundii*, and *Escherichia intermedium* were very sensitive test organisms.

These indications of antibacterial substances in the edible pulp of bananas suggested a study of the growth of anaerobic spoilage organisms in banana purée. Some research on bananas involving strains of Type A and Type B *Clostridium botulinum* has been reported by Townsend and associates *(13)*. It is the purpose of this paper to report on the effect of banana purée on the growth of the additional spoilage organisms, *Bacillus coagulans*, Thermophilic Anaerobe No. 3814, Putrefactive Anaerobe No. 3679, as well as *Clostridium botulinum* No. 62A and *Clostridium botulinum* No. 213B.

EXPERIMENTAL

Basic experimental plan. Cells of spoilage bacteria were dispersed in sterile banana purée, the mixtures were sealed in containers under vacuum, and periodic bacterial

[a] This report is based on part of the Ph.D. thesis presented in the Department of Food Technology, Massachusetts Institute of Technology, by E. N. Bilenker in 1955. Contribution No. 366 from the Department of Food Technology, M.I.T.

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colony counts were made on the inoculated samples after storage at specified temperatures. The counts were made on the day the purée was inoculated and after 1 week, 4 weeks, and 8 weeks. A marked incerase in the colony counts, if obtained during the incubation periods, would indicate a lack of antibacterial activity against the particular species of spoilage organism.

Banana purée. Banana purée, provided by the United Fruit Company, was prepared (according to processing records) from ripe banana pulp only, with no added material such as sugar or ascorbic acid. The purée was made by a modification of the Martin *(5)* flash sterilization process. Bananas at the color 6 stage of ripeness were peeled and then reduced to pumpable consistency. Next, the banana purée was pumped through equipment that quickly raised it to a sterilizing temperature. It was held at the sterilizing temperature for a critical number of seconds and then rapidly cooled. The sterile purée was packed under aseptic conditions. All the purée used in this experiment was from one production run and possessed a pH of 4.8.

Sterility checks on fluid thioglycollate medium (Bacto B256) and colony counts showed that the purée was either sterile or developed no colonies when plated at very low dilutions.

Bacterial suspensions. The 5 strains of spoilage bacteria used were obtained from the National Canners Association collection.

A stock suspension of *Bacillus coagulans* was heated at 80° C for 12 min. Serial dilutions were plated out on Bacto Thermoacidurans agar *(12)*. After incubation for 24 hr at 55° C, discrete colonies were subcultured on slants of the same medium. After incubation for 48 hr at 55° C, the growth from each slant was used to inoculate larger slants consisting of Thermoacidurans agar. The large slants were incubated at 55° C for 7 days; microscopic examination of smears, stained with the malachite green spore stain technique, showed a fair amount of spores. A 5-ml portion of sterile water was added to each slant. The slants were tilted several times during a 30-min period to wet the entire surface growth. The growth was scraped off and suspended with the aid of a bent inoculating needle. The tube suspensions were pooled in a 100-ml Escher-stoppered dilution bottle containing glass beads. After vigorous shaking for 1 min, the suspension was filtered through loosely-packed sterile glass wool into a sterile 2-oz screw cap jar containing glass beads. A 2-ml portion of the suspension was heated at 80° C for 15 min; serial dilutions then were made from the heated and from an unheated portion for plate counts on Bacto Thermoacidurans agar. After incubation for 48 hr at 55° C, duplicate counts averaged as shown in Table 1.

TABLE 1

Colony counts of the bacterial cell suspensions prior to refrigerator storage

Microorganism	Heat treatment of spore suspension		Incubation		Colonies per ml of suspension	
	Temp., °C	Time (min)	Temp., °C	Time (hr)	Heated (Spores)	Unheated Spores and vegetative cells
Bacillus coagulans	80	15	55	48	1,200,000	20,000,000
Thermophilic Anaerobe No. 3814	80	15	55	72	450,000	350,000
Putrefactive Anaerobe No. 3679	80	15	37	48	7,000,000	4,400,000
Clostridium botulinum No. 62A	80	20	30	24	300,000,000	400,000,000
Clostridium botulinum No. 213B	80	20	30	24	150,000,000	100,000,000

A Thermophilic Anaerobe No. 3814 cell suspension was prepared according to the method of Reed *et al (8)*, with the modifications that the procedure was scaled down to yield a bacterial cell crop in 500 ml instead of in 6 liters of liver broth, and the final incubation at 42° C was for 30 days instead of for 10 days. An aliquot part of the final flask contents was tested for viable spores after it had been heated at

80° C for 15 min and inoculated into a liver broth tube containing pieces of liver and iron nails. Vigorous growth was apparent after incubation for 72 hr at 55° C. The final centrifuged and filtered cell suspension was placed under refrigeration in a 2-oz screw cap jar containing glass beads. An aliquot part was heated at 80° C for 15 min; bacteria in it and in an unheated portion of the Thermophilic Anaerobe

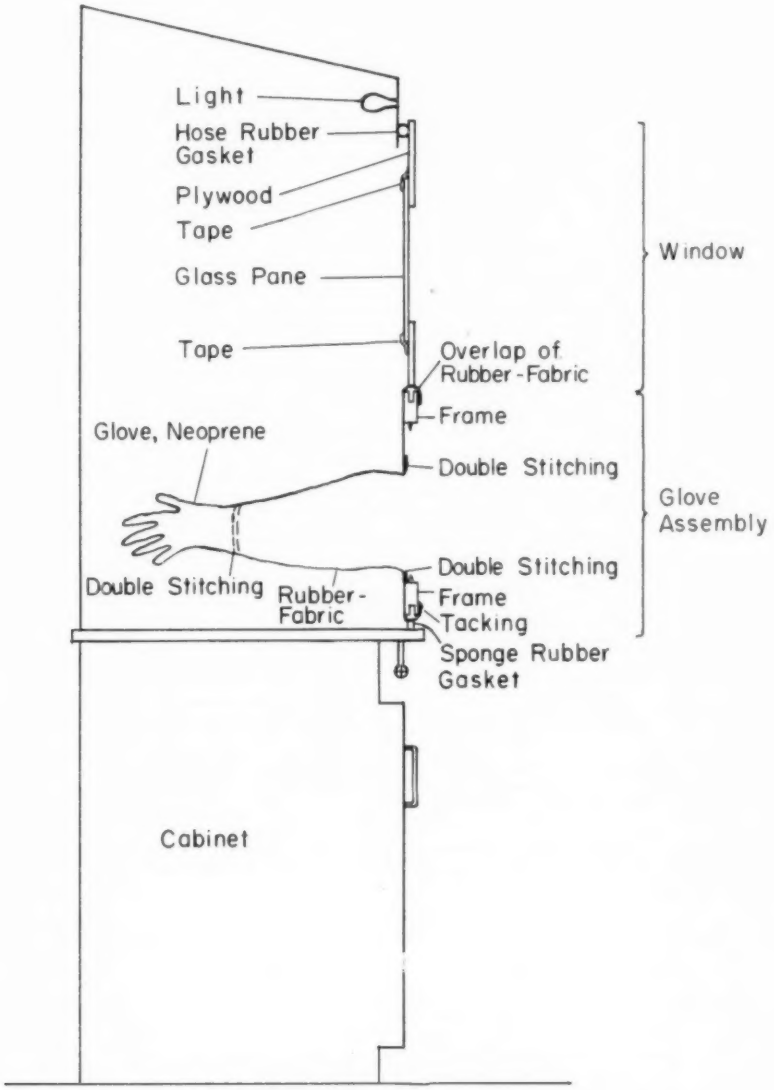

Figure 1.

No. 3814 cell suspension were determined, with the use of Norite agar in anaerobic culture tubes. The results for the bacterial suspensions are shown in Table 1.

Cultivation of the Putrefactive Anaerobe No. 3679 cell suspension was done according to the method of Reed *et al (8)* with the modifications that the procedure was scaled down to a final volume of one liter, instead of 5 liters, of pork broth, and that the final incubation period was for one week, instead of two, at 30° C. The centrifuged and filtered cell suspension was stored under refrigeration in a 2-oz screw cap jar containing glass beads. An aliquot part of the cell suspension was heated at 80° C for 15 min; this and an unheated portion were examined for cell counts on Andersen's medium *(1)* in anaerobic culture tubes. The colonies were counted as described in the preceding paragraph, with results as shown in Table 1.

Cultivation of the *Clostridium botulinum* No. 62A and No. 213B cell suspensions was accomplished by a modification of the technique of Sognefest and co-workers *(11)*.

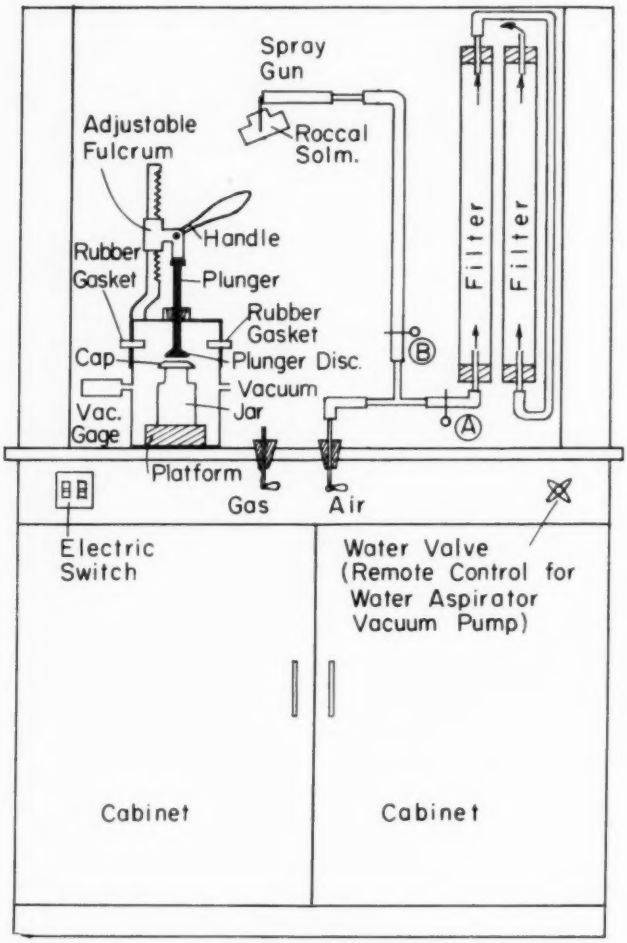

Figure 2.

The contents of an Erlenmeyer flask (120 ml of beef heart infusion, beef heart particles, and iron strips) were inoculated and incubated for 24 hr at 37° C. The broth culture thus obtained was strained through cheese cloth into centrifuge bottles and centrifuged. The cells were re-suspended in a small portion of the culture fluid and filtered through sterile glass wool into a sterile bottle containing glass beads. The suspension was stored in a refrigerator. Colony counts were made on Andersen's medium in anaerobic tubes; incubation was at 30° C for 24 hr. Bacteria from both heat-treated (80° C, 20 min) and unheated aliquot portions were counted as reported in Table 1.

Aseptic chamber. The experimental procedure for bacterial colony counts involved the periodic opening, sampling, and resealing of each vacuum-packed jar of inoculated banana purée. Ordinarily, each breaking of the vacuum seal increases the possibility of contaminating the sample with airborne microbes. In this investigation, this hazard was reduced by performing all banana purée sampling operations inside an aseptic chamber supplied with filtered air.

The aseptic chamber was made inexpensively by modifying a chemical fume hood. The front movable window was removed, and all joints in the hood interior were sealed with drafting tape. The hood water spout was fitted with a water aspirator; the water outlet of the aspirator was extended by means of rubber hose and glass tubing, through a one-hole rubber stopper which was used to plug the hood sink drain.

The glove assembly (Fig 1) was designed with generous proportions to permit access to any part of the aseptic chamber with either gloved hand. The assembly was fabricated from a pair of Neoprene rubber gloves and rubberized fabric sheeting. The latter was cut to provide 2 conical sleeves and a sheet section with 2 holes in it to receive the sleeves. Rubberized parts were sewn together (on a domestic sewing machine) using at least two rows of stitching throughout. The entire glove assembly was tacked onto a wooden frame hinged in the center. The wooden frame was made of pieces of tongue and groove lumber, with the tongues outward on the vertical sides and the grooves outward on the top edge. The side edges of the frame fitted snugly into the hood window sash, and the horizontal groove construction formed a good joint with the plywood of the upper window frame (Fig. 1).

The window was constructed of a plywood frame and plain window glass, sealed to the wood with drafting tape. The window assembly remained in the hood window sash at all times, whereas the glove assembly was removable.

Before its installation as an aseptic chamber fixture, the air-filtering assembly (Fig. 2) was sterilized, in its entirety, in an autoclave using steam under pressure. The inverted beaker (at the air exit port) and the valve at A in Fig 2 protected the filter system (columns packed with cotton) when not in use. The spraying device was a hand-pump insecticide gun adapted for the purpose. Roccal (alkyldimethylbenzylammonium chlorides) was used as a germicidal spray.

Clostridium botulinum. The studies of *Clostridium botulinum* were carried out in essentially the same way as those with the other bacterial species. Two exclusive features of the *Clostridium botulinum* studies were the use of a neutralized banana purée for spore inoculation and toxicity tests with mice.

The neutralized banana purée was prepared by mixing about 300 g of the regular canned purée with 30 ml of a sterile sodium bicarbonate solution in a sterile blender bowl. The bicarbonate salt was used as the neutralizing agent because it favors the breaking of spore dormancy by enriching the banana purée substrate with the bicarbonate ion (1). One batch of neutralized purée had a pH of 6.86; part of this was inoculated with a heated spore suspension of *Clostridium botulinum* No. 62A. A second batch of neutralized purée was used to make a corresponding set of uninoculated, neutral purée controls.

To obtain information relating to possible toxin production by *Cl. botulinum*, a limited number of toxitity tests were made on some of the purée samples 6 weeks after they were inoculated with spores. Mice were used because of their extreme sensitivity to very small amounts of toxin. Results of the tests are summarized in Table 2.

Samples of banana purée for mouse inoculation, including the control, were all from the 37° C incubator. A product for injection purposes was prepared by shaking 5 to 10 g of purée with 20 ml of sterile normal saline in a vial containing glass beads. The material was then allowed to settle and the turbid supernatant was taken for

TABLE 2

Mouse tests to detect the production of toxin in sterilized banana purée which had been inoculated with the spores of Clostridium botulinum[1]

Treatment of mouse			
Mouse serial number	Injected with antitoxin	Material injected into mouse	Results
1	0.3 ml	62A inoc. purée	Survived
2	0.3 ml	213B inoc. purée	Survived
3	None	Pure culture of 62A	Died within 20 hr
4	None	Pure culture of 213B	Died within 20 hr
5	None	Plain, control purée[2]	Survived
6	None	62A inoc. purée	Survived
7	None	213B inoc. purée	Survived
8	None	None	Survived

[1] Tests were carried out 6 weeks after the samples were inoculated with the spores of *Cl. botulinum*. The material was not heat treated to inactivate toxin.
[2] This sample of purée was not inoculated with *Cl. botulinum*.

injection. Each mouse (Table 2) received 0.4 ml of purée suspension in the form of an intraperitoneal injection. Jenson-Salsbery Polyvalent *Clostridium botulinum* Antitoxin was used on 2 mice in the amount of 0.3 ml each; the antitoxin was injected subcutaneously 2 hr before the intraperitoneal injection of the indicated (Table 2) purée suspension. Two mice were injected with the broth from pure cultures of the 2 strains of *Clostridium botulinum*. Each mouse was kept in its own cage with adequate rations. The 5 surviving mice were observed periodically for 11 days, during which time they showed no signs of intoxication.

Colony count methods. The cultural and incubation conditions used for counting the bacteria in the banana purée samples are summarized in Table 3. Counts involving Petri dishes were made with the aid of a Quebec Colony Counter.

TABLE 3

Summary of the methods used to obtain the bacterial colony counts of banana purée

Bacterial species	Agar medium (ref)	Incubation	
		Temperature, °C	Period (hours)
Bacillus coagulans	Thermoacidurans *(12)*	55	48
Thermophilic Anaerobe No. 3814	Norite-Tryptone *(8)*	55	72
Putrefactive Anaerobe No. 3679	Andersen's *(1)*	30	48
Clostridium botulinum No. 62A	Andersen's *(1)*	30	48
Clostridium botulinum No. 213B	Andersen's *(1)*	30	48

RESULTS AND DISCUSSION

The bacterial colony counts of banana purée samples inoculated with *Bacillus coagulans* cell suspensions are given in Table 4. The banana purée supported the multiplication of *Bacillus coagulans* at 20° C, 30° C and 55° C. It appears that the growth in the purée incubated at 30° C was highest. At 55° C the observed colony counts were much lower, but it is possible that at the higher temperature accelerated activity might have occurred and the population crested and declined before the one-week colony counts were taken.

At the 20° C and 30° C storage temperatures, the purées inoculated with the heated cell suspension ("spores" in Table 4) had higher 8-week colony counts than the purées inoculated with the unheated suspension of bacterial

TABLE 4

Periodic bacterial colony counts of banana purée samples inoculated with Bacillus coagulans

Storage temp °C	Weeks in storage	Average bacterial colonies per gram			
		Inoculated with spores		Inoculated with spores and vegetative cells	
		Heavy	Light	Heavy	Light
20	0	9,200	500	52,000	300
	1	37,000	2,800	530,000	3,500
	4	1,600,000	14,000	250,000	6,100,000
	8	520,000	400,000	490	220
30	0	9,200	500	52,000	300
	1	30,000,000	37,000,000	18,000,000	79,000,000
	4	1,100,000	3,100,000	12,000	260,000
	8	7,100	12,000	500	850
55	0	9,200	500	52,000	300
	1	110,000	160,000	840	130,000
	4	0	0	0	0
	8	0	0	0	0

spores and vegetative cells. The 8-week colony counts in the purées initially inoculated with "light" suspensions were approximately the same as the counts in the purées inoculated with "heavy" suspensions of the same category.

The pH values of the inoculated and uninoculated (control) samples of banana purée held at 3 different temperatures for 4½ months are listed in Table 5. After this period, the colors of all samples (including the controls) were considerably browner than the color of the purée in a freshly opened No. 10 can; 55° C samples had a light tan color. Inoculation with *Bacillus*

TABLE 5

The pH of banana purée samples inoculated with Bacillus coagulans and held for 4½ months at various temperatures

Storage temp °C	Original inoculum	pH	Maximum observed [1] colony count per gram
20	Heavy; spores	4.59	1,600,000
	Light; spores	4.59	400,000
	Heavy; spores & veg. cells	4.68	530,000
	Light; spores & veg. cells	4.68	6,100,000
	None	4.84	0
30	Heavy; spores	4.41	30,000,000
	Light; spores	4.50	37,000,000
	Heavy; spores & veg. cells	4.41	18,000,000
	Light; spores & veg. cells	4.43	79,000,000
	None	4.72	0
55	Heavy; spores	4.10	110,000
	Light; spores	4.18	160,000
	Heavy; spores & veg. cells	4.10	840
	Light; spores & veg. cells	4.10	130,000
	None	4.20	0

[1] Data taken from Table 4.

coagulans was associated with the development of a pH lower than that of the uninoculated control. A comparison of the 20° C and 30° C samples shows higher counts accompanied by lower pH values at the 30° C temperature.

The inoculated samples (20° C and 30° C) had better color than their corresponding controls; this might have been due to the lower pH of the inoculated samples.

The bacterial colony counts of banana purée samples inoculated with Thermophilic Anaerobe No. 3814 (Table 6) show that at 20° C and 30° C

TABLE 6

Periodic bacterial colony counts in banana purée samples inoculated with Thermophilic Anaerobe No. 3814

Storage temp °C	Weeks in storage	Average bacterial colonies per gram			
		Inoculated with spores		Inoculated with spores and vegetative cells	
		Heavy	Light	Heavy	Light
20	0	670	280	890	190
	1	1,100	300	620	130
	4	2,100	550	520	320
	8	6,600	750	1,000	600
30	0	670	280	890	190
	1	1,200	290	350	180
	4	1,600	360	1,100	450
	8	2,400	650	830	330
55	0	670	280	890	190
	1	360	0	140	0
	4	0	0	0	0
	8	0	0	0	0

TABLE 7

The pH of banana purée samples inoculated with Thermophilic Anaerobe No. 3814 and held for 3 months at various temperatures

Storage temp °C	Original inoculum	pH	Maximum observed [1] colony count per gram
20	Heavy; spores	4.81	6,600
	Light; spores	4.81	750
	Heavy; spores & veg. cells	4.52	1,000
	Light; spores & veg. cells	4.52	600
	None	4.79	0
30	Heavy; spores	4.76	2,400
	Light; spores	4.73	650
	Heavy; spores & veg. cells	4.72	1,100
	Light; spores & veg. cells	4.76	450
	None	4.78	0
55	Heavy; spores	4.35	670
	Light; spores	4.36	280
	Heavy; spores & veg. cells	4.39	890
	Light; spores & veg. cells	4.39	190
	None	4.38	0

[1] Data taken from Table 6.

banana purée supported a condition varying between bacteriostasis and that permitting a very slight increase in the population of this anaerobe.

The pH values (Table 7) are, with two exceptions, very close for both the inoculated purée samples and their corresponding controls. The exceptions are the two 20° C samples that had a pH of 4.52. (These lower pH values may not be explained in terms of bacterial acid production because higher colony counts in other inoculated samples were not accompanied by a lowered pH.)

The samples inoculated at 20° C tended toward a grey-colored darkening, whereas the 30° samples had a tan cast. The samples at 55° C had a scorched odor and chocolate brown color. None of the stored samples possessed the lighter color of the banana purée present in a freshly opened No. 10 tin.

The bacterial colony counts in Table 8 show that the population of Putrefactive Anaerobe No. 3679 tended to remain the same in any given set of the inoculated samples of banana purée.

TABLE 8

Periodic bacterial colony counts in samples of banana purée inoculated with Putrefactive Anaerobe No. 3679

Storage temp °C	Weeks in storage	Average bacterial colonies per gram			
		Inoculated with spores		Inoculated with spores and vegetative cells	
		Heavy	Light	Heavy	Light
20	0	120,000	5,100	96,000	3,400
	1	67,100	5,100	120,000	3,900
	4	73,000	5,400	130,000	4,900
	8	61,000	3,700	130,000	3,100
30	0	120,000	5,100	96,000	3,400
	1	89,000	4,300	120,000	2,800
	4	110,000	2,400	100,000	4,300
	8	68,000	2,800	170,000	3,000
37	0	120,000	5,100	96,000	3,400
	1	90,000	4,400	150,000	3,600
	4	130,000	3,500	200,000	4,000
	8	94,000	2,600	110,000	2,700

There were no marked changes in population that could be associated with the choice of storage temperature or the nature of the inoculum used for the Putrefactive Anaerobe No. 3679 cell suspension.

The pH values of the banana purée samples inoculated with Putrefactive Anaerobe No. 3679 (Table 9) are nearly the same within each storage temperature category; this reflects a static condition.

The bacterial colony counts of banana purée samples inoculated with *Clostridium botulinum* No. 62A (Table 10) indicate that there were no significant changes in the population of this organism during storage in the plain, unneutralized banana purée.

The mouse (Serial No. 6 in Table 2) that was injected with purée inoculated with a suspension of *Clostridium botulinum* No. 62A survived. This indicated that any population increase or metabolic activity by *Cl. botuli-*

num in banana purée under the conditions of the test, was not accompanied by a significant quantity of toxin production. The purée used for the mouse injection had been inoculated with spores and incubated for six weeks at 37° C before its use for the animal test.

TABLE 9

The pH of banana purée samples inoculated with Putrefactive Anaerobe No. 3679 and held for 2½ months at various temperatures

Storage temp °C	Original inoculum	pH	Maximum observed [1] colony counts per gram
20	Heavy ; spores	4.82	120,000
	Light ; spores	4.82	5,400
	Heavy ; spores & veg. cells	4.82	130,000
	Light ; spores & veg. cells	4.82	4,900
	None	4.82	0
30	Heavy ; spores	4.75	120,000
	Light ; spores	4.73	5,100
	Heavy ; spores & veg. cells	4.78	170,000
	Light ; spores & veg. cells	4.73	4,300
	None	4.74	0
37	Heavy ; spores	4.72	130,000
	Light ; spores	4.70	5,100
	Heavy ; spores & veg. cells	4.72	200,000
	Light ; spores & veg. cells	4.70	4,000
	None	4.71	0

[1] Data taken from Table 8.

TABLE 10

Periodic bacterial colony counts in banana purée samples inoculated with Clostridium botulinum No. 62A

Storage temp °C	Weeks in storage	Average bacterial colonies per gram		
		Neutralized purée inoculated with spores	Plain banana purée	
			Inoculated with spores	Inoculated with spores and vegetative cells
20	0	87,000	52,000	150,000
	1	23,000	50,000	110,000
	4	32,000	64,000	100,000
	8	12,000	45,000	170,000[3]
30	0	87,000	52,000	150,000
	1	310,000	89,000	150,000
	4	140,000	56,000	140,000
	8	60,000	75,000	200,000
37	0	87,000	52,000	150,000
	1	60,000[1]	100,000	170,000
	4	47,000	130,000	96,000
	8	11,000[2]	97,000	300,000

The plain uninoculated purée control samples showed no growth at the 0-, 1-, 4-, and 8-week intervals.

[1,2] The neutralized uninoculated puree control samples showed no growth at the test intervals, with the exception of the controls corresponding to the [1] and [2] samples. Examination of the control sample corresponding to [1] disclosed a colony of *ca* 250,000 per gram; examination of the [2] control disclosed a count of 4,100 per gram.

[3] A small, mold-like colony was noted on the surface of the purée when its jar was opened in the aseptic chamber.

The neutralized banana purée did not support an increase in the cell count of *Cl. botulinum* No. 62A during the eight-week test period.

Neither the choice of bacterial inoculum nor the temperature at which each set of samples was held had any significant effect on the periodic cell counts.

Data in Table 11 show little change in the bacterial population of plain banana purée inoculated with *Clostridium botulinum* No. 213B.

TABLE 11

Periodic bacterial colony counts in banana purée samples inoculated with Clostridium botulinum No. 213B

Storage temp °C	Weeks in storage	Average bacterial colonies per gram		
		Neutralized purée inoculated with spores	Plain banana purée	
			Inoculated with spores	Inoculated with spores and vegetative cells
20	0	67,000	63,000	14,000
	1	35,000	35,000	20,000
	4	260,000	31,000	25,000
	8	14,000	45,000	20,000
30	0	67,000	63,000	14,000
	1	24,000	23,000	17,000
	4	170,000	19,000	22,000
	8	15,000	16,000	14,000
37	0	67,000	63,000	14,000
	1	13,000	38,000	22,000
	4	10,000	25,000	13,000
	8	2,100	14,000	8,700

The plain purée inoculated with spores was injected into a mouse after the purée had been incubated for 6 weeks at 37° C. The mouse (Serial No. 7 in Table 2) survived. This indicated that no significant amount of toxin from *Clostridium botulinum* No. 213B was in the purée sample at the time the purée was used for mouse injection.

The neutralized banana purée inoculated with *Clostridium botulinum* No. 213B and incubated at 20° C and 30° C showed population increases at the 4-week test; no such increase was observed in a portion of the same purée incubated at 37° C. By the eighth week, the bacterial colony counts in all the inoculated, neutralized purée samples were less than the initial colony count (0-week count).

The antibacterial effect of banana purée varied with the species of spoilage bacteria. The growth-retarding effect on all but *Bacillus coagulans* may have been ascribable to the pH. This possibility was considered experimentally by neutralizing the banana purée prior to its inoculation with the 2 strains of *Clostridium botulinum;* the bacteriostatic effect of the banana purée persisted despite neutralization. These negative results with the neutralized banana purée seem to differ from the findings of Townsend *et al (13)*. This variance may be explained, possibly, by noting that they used, for one series of inoculations, a composite of several Type A strains of *Clostridium botulinum*, and for another, a composite of several Type B strains. This present investi-

gation was limited to only 2 strains of *Clostridium*, namely 62A and 213B. The neutralized banana substrates were also different. The Townsend purée was more severely treated by autoclaving at 121° C for 20 minutes.

In addition to the pH, the bacteriostatic effect on all but *Bacillus coagulans* may have been the result of the peculiar nutritional requirements of the spoilage bacteria which could not be satisfied by banana purée. Antibacterial banana substances possibly might have been involved, but the experimental results were insufficient for separating nutritional from antibacterial considerations.

SUMMARY

The effect of banana pulp on the growth of spoilage bacteria was tested by making periodic bacterial counts on samples of banana purée inoculated with various spoilage organisms. Banana purée supported vigorous growth of *Bacillus coagulans* but inhibited the growth of other species tested, i.e., Thermophilic Anaerobe No. 3814, Putrefactive Anaerobe No. 3679, and *Clostridium botulinum* Nos. 62A and 213 B. The growth of both strains of *Clostridium botulinum* was inhibited in neutralized as well as in untreated banana purée under the conditions of the experiments.

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RADIATION STERILIZATION OF FOOD. I. PROCEDURES FOR THE EVALUATION OF THE RADIATION RESISTANCE OF SPORES OF *CLOSTRIDIUM BOTULINUM* IN FOOD PRODUCTS[a]

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Establishment of radiation processing requirements for commercial sterilization of food products presupposes a sound and reasonably accurate estimate of the maximum resistance to radiation of spores of *Clostridium botulinum*. Schmidt, in 1957, cited by Niven *(7)* estimated a radiation dose of 4.5 megarad would be required to be equivalent in safety to thermally processed foods. This estimate was based upon published and unpublished estimates then current of the resistance of spores of *Cl. botulinum;* the data supporting these estimates were, admittedly, meagre. Since quality considerations and cost are related to the dose level requirement, the final dose recommendation should be sufficiently high to be equivalent in safety to thermally processed foods, but not excessively high in terms of this standard. For these reasons, extensive knowledge of the resistance of spores of a large number of strains of *Cl. botulinum* in various food products will be required.

Data upon the radiation resistance of spores of *Cl. botulinum* in various substrates have been published by Morgan and Reed *(6)*, Kempe, Graikoski and Gillies *(5)*, Kempe *(4)*, Wagenaar and Dack *(10)*, Denny *et al (2)* and Pratt *et al (8)*. In 1957 an opportunity was presented to the authors to study the radiation resistance of spores of *Cl. botulinum* in a series of food products of the types to be sterilized for human feeding tests.[b]

The purpose of this paper is to describe the procedures used for the evaluation of the radiation resistance of spores of *Cl. botulinum* in food products and to present and discuss the results.

EXPERIMENTAL PROCEDURE

Products. The following products were received in No 10 cans, packed commercially under Quartermaster Food and Container Institute supervision: roast pork loin, cooked steak, roast chicken parts, and green beans. The green beans had been blanched and frozen; the other products were received refrigerated within 24 hr after packing. Whole kernel corn in brine was also used in these experiments to provide for a cross-over comparison with data obtained previously with a suspension tested in corn and other products.

Packing. Products were repacked in 202 x 202 "C" enameled cans. The pork loin and steak were cut into small pieces, approximately 1.0 x 0.5 x 0.5 inches for repacking. Meat and skin of chicken parts were stripped from the bones and cut into pieces no larger than 1.0 x 0.5 x 0.5 in. In repacking the chicken, each can received a mixture

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[b] Acknowledgment, with thanks, is due to personnel of the Quartermaster Food and Container Institute for the Armed Forces for cooperation and the provision of food product samples, and for providing the required irradiation.

of light and dark meat and skin. The green beans were thawed and cut into lengths of 1.0 in to 1.5 in for repacking. The whole kernel corn was thawed and packed with brine. The following fill-in weights in grams were used: pork loin 80, steak 80, chicken parts 80, green beans 37, and whole kernel corn in brine, corn 56, brine 36 (ml). Each can was check weighed for uniform fill-in weight.

Inoculation and closing. Each can was inoculated with 1 ml of a suspension of spores of *Cl. botulinum* types A and B, described hereafter. An inoculum level of 100 million spores per can was used. For all products, except whole kernel corn in brine, a 1 ml tuberculin syringe with a 2 in 18-gauge needle was used. The inoculum was introduced both on the surface and slightly into the tissue of each product. For the whole kernel corn the spores were inoculated into the brine using a B-D syringe pipette. After inoculation the cans were closed on a vacuum closing machine with at least 20 in machine vacuum and packed in cracked ice for chilling. The inoculated cans were held overnight at 38–40° F.

Irradiation. The morning following packing and inoculation, the cans were transported in cracked ice to Argonne National Laboratories, Lemont, Illinois, and held refrigerated until irradiated. Dosimetry and calculation of exposure times to give the desired radiation dose were performed by Argonne personnel. Just prior to irradiation, each lot of 10 cans was warmed in water to approximately 80° F. Cans were placed in polystyrene foam holders and irradiated by the procedures described by Pratt *et al (8)* designed to equalize the dose received by each can. Following irradiation, cans were held at room temperature 1 or 2 days until returned to the laboratory for incubation at 85° F.

Observation for swells. Incubated cans were observed for swells every 3 or 4 days following incubation in order to record the rate of development of survivors.

Toxin assay. Each swelled can, except whole kernel corn in brine, was chilled in the refrigerator, then opened aseptically, and contents transferred to a sterile aluminum Waring blender with a threaded screw cap. Sufficient sterile water was added to give a final dilution of 1–5. After blending, a 5 ml sample was transferred to a sterile cotton-plugged 13 x 100 mm tube. A second 20 ml sample was transferred to a sterile 20 x 150 mm screw cap tube and refrigerated as a retained sample. Swelled cans of whole kernel corn and brine were sampled for brine only, without blending. The 5 ml samples were centrifuged at 6000 rpm in a Servall XL centrifuge. For toxin assay, 0.25 ml of each supernatant was injected intraperitoneally into a 20 g white mouse. If the mouse survived 4 days, 2 additional mice were injected with 0.5 ml of the same supernatant. Only if these 2 mice survived was the supernatant recorded as nontoxic. All toxic samples were confirmed by protection and typing tests using three mice: one unprotected, one protected with Type A antitoxin and one protected with Type B antitoxin.

Toxin assay and spore recovery on unswelled cans. After 12–15 months incubation, the 20 cans at the lowest irradiation level not showing swells in each product were examined for toxicity and viable spores. The sample from each can was prepared as described for toxin assay. In addition to toxin assay, two 5 ml samples of the blend were transferred to 20 ml of freshly heated liver broth with liver particles in 20 x 150 mm screw cap tubes. Tubes were incubated at 85° F for 2 months. Tubes showing evidence of growth were examined microscopically, toxin assayed and typed.

Preparation of spore suspensions. The following cultures, received from Dr. Gail M. Dack, University of Chicago, were used: Type A: 62, 109 and T; and Type B: 169 and 126.7. The medium of Wagenaar and Dack *(11)* was used for the production of spore crops. The medium consisting of 5% Trypticase (BBL) and 0.5% Peptone (Difco) was adjusted to pH 7.0, distributed 150 ml per 8 oz screw cap prescription bottle and sterilized 15 min at 250° F. Stock cultures in the same medium in screw cap tubes were used as inocula. Just prior to inoculation each bottle of medium received 1.5 ml of a 10% solution of sodium thioglycollate, sterilized 5 min at 250° F, to give 0.1% final concentration. For each strain, 2 bottles were each inoculated with 1 ml of stock culture and incubated at 85° F for 6 days. Cultures were centrifuged at 2000 rpm and the sediment of each strain resuspended in 60 ml of sterile water to give a five-fold concentration for each culture. These suspensions were then refrigerated as stock suspensions. Total and viable spore yields of the suspensions prepared in this way were considerably lower than with the spore inoculum and inversion mixing

procedure described by Schmidt *(9)*; however, the yields were sufficient for the purposes of this experiment.

Standardization of suspensions. Suspensions were standardized for total spore count using a Petroff-Hauser Chamber and dark phase microscopy. With total spore count as a guide, dilutions were prepared and viable spore count determined from the colony count in Pork-Pea Infusion Agar, Anderson *(1)*, incubated at 85° F following a heat shock of 13 min at 176° F. Procedures for total and viable count are described more completely by Schmidt *(9)*.

Preparation of dilute suspensions for inoculation. Based upon the viable count of the stock suspensions, aliquots of each were preheated 13 min at 176° F, combined and diluted with sterile water to give a final concentration of 100 million per ml (20 million per ml of each strain). The concentration of this suspension was verified by viable count.

Calculation of results. In addition to recording the results as survival and destruction dose, the partial spoilage data were evaluated in terms of a radiation D value (dose for 90% destruction, calculated upon the assumption of an approximately logarithmic rate of destruction over the major portion of the 9–10 log cycle reduction which occurs to reduce the inoculum to the point of partial spoilage). With this assumption the following equation may be used:

$$D = \frac{\text{Radiation dose (megarad)}}{\text{Log M} - \text{Log S}}$$

where M = the total inoculum; that is, the spores per container times the number of containers

S = the number of swelled containers assuming one survivor per container

RESULTS AND DISCUSSION

Results of spoilage, toxin assay and spore recovery in the various products are presented in Tables 1 and 2. It should be noted in Table 1 that the resistance in green beans was determined from an initial spore load of 10 million per can instead of 100 million. This was necessary in order to provide sufficient of the mixed suspension to complete the work in green beans. As shown in Table 2, a test was run with one million spores per container as well as with the 100 million spore load in whole kernel corn in brine.

Under toxicity the symbol T indicates: toxin detected in 0.25 ml; WT—weakly toxic—no toxin in the 0.25 ml assay, but toxicity was demonstrated in at least one of the two 0.5 ml injections; and NT indicates no toxin detectable in repeated 0.5 ml injections. Two weakly toxic and 3 non-toxic spoilage samples were encountered. In other unpublished work from this laboratory, more weakly toxic samples have been encountered. In view of this we currently favor the preparation of a 1:1 blend for toxin assay and spore recovery. Two of the nontoxic spoilage samples, chicken parts and corn, gave highly toxic cultures when samples equivalent to a 0.2 mg of original spoiled contents were subcultured into PE2 medium, Folinazzo and Troy *(3)*.

The culture isolated from the spoiled pork loin has given anomalous results. The original isolation in PE2 medium was nontoxic as were two further transfers in PE2. A transfer into a tube of the trypticase-peptone medium was nontoxic; however, when bottles of this medium were inoculated with heat-shocked spores from this tube in order to prepare a suspension of the "nontoxic" strain, toxin was demonstrable in 0.25 ml quantities of the supernatant and in subcultures of the spore suspension into PE2 medium.

TABLE 1

Spoilage, toxicity and spore recovery in 202 x 202 cans of various food products inoculated with spores of Cl. botulinum and irradiated to various dose levels

Product	Dose megarad	Spoilage [1]	Toxicity of swelled cans			Unswelled cans [1]	
			T [2]	WT [3]	NT [4]	Toxic	Spore recovery
Chicken parts	2.51	7/20	6	0	1	..	..
	2.79	4/20	4	0	0	..	..
	3.07	0/20	..	..	..	0/20	0/20
Beef steak	2.51	7/20	7	0	0	..	..
	2.79	2/20	2	0	0	..	..
	3.07	0/20	..	..	..	0/20	0/20
Pork loin	2.14	18/20	17	1	0	..	..
	2.42	5/20	3	1	1	..	..
	2.70	0/20	..	..	..	0/20	0/20
	3.07	0/20	..	..	..	..	..
Green beans [5]	1.30	6/20	6	0	0	..	..
	1.49	0/20	..	..	..	0/20	3/20
	1.86	0/20	..	..	..	0/20	0/20
	2.04	0/20	..	..	..	..	..

[1] Number found/number tested
[2] T—Toxic
[3] WT—Weakly toxic
[4] NT—Nontoxic
[5] 10,000,000 spores per can

TABLE 2

Spoilage, toxicity and spore recovery of whole kernel corn in 202 x 202 cans inoculated with spores of Cl. botulinum and irradiated to various dose levels

Inoculum level per can	Dose megarad	Spoilage [1]	Toxicity of swelled cans			Unswelled cans [1]	
			T [2]	WT [3]	NT [4]	Toxic	Spore recovery
10^8	2.32	20/20	20	0	0	..	..
	2.70	9/20	9	0	0	..	..
	3.07	0/20	..	..	..	0/20	0/20
10^6	1.86	12/20	12	0	0	..	..
	2.04	7/20	6	0	1	..	..
	2.32	1/20	1	0	0	..	..

[1] Number found/number tested
[2] T—Toxic
[3] WT—Weakly toxic
[4] NT—Nontoxic

Studies of cultures isolated from nontoxic spoilage are being continued. All cultures isolated appeared to be uncontaminated typical clostridia and in each case when toxin was produced it was identified as Type A. The toxic spoilage in all products was typed and in every instance only Type A toxin was found.

No toxin was found in any of the unspoiled cans of any product at the first radiation level showing no spoilage. With the exception of 3 cans of green beans at 1.49 megarad, no viable spores were found in the unspoiled flat cans at the first level of radiation showing no spoilage. No spores were recovered from the second level of radiation showing no spoilage in green beans. The cultures isolated from the unspoiled flat cans of green beans produced Type A toxin.

TABLE 3

Calculation of radiation D values from spoilage in chicken parts, beef steak, pork loin and green beans

Product	Dose megarad	Spoilage [1]	D value (megarad)		
			Log M = 9.3	Log M = 9.08	Log M = 8.6
Chicken parts	2.51	7/20	0.30	0.30	0.32
	2.79	4/20	0.32	0.33	0.35
Beef steak	2.51	7/20	0.30	0.30	0.32
	2.79	2/20	0.31	0.32	0.34
Pork loin	2.14	18/20	0.27	0.27	0.29
	2.42	5/20	0.28	0.29	0.31
			Log M = 8.3	Log M = 8.08	Log M = 7.6
Green beans [2]	1.30	6/20	0.17	0.18	0.19

[1] Number found/number tested
[2] 10,000,000 spores per can

Radiation D values calculated from the partial spoilage data are shown in Tables 3 and 4. For an initial inoculum of 100 million spores per can with 20 can lots, Log M = 9.3. However, since no Type B toxin has been produced in any of the spoilage samples, it is assumed that the Type B portion of the inoculum had not produced any survivors. Therefore, with respect to the spoilage produced, only the Type A portion of the inoculum should be considered for purposes of calculation. We can assume either that survivors occurred at random from each of the Type A strains or that all survivors originated from one Type A strain. If survivors occurred at random from each of the Type A strains then the initial effective inoculum would be 60 million per can and Log M = 9.08. If all the spoilage arose from only one Type A strain then the initial effective inoculum would be 20 million per can and Log M = 8.6.

In Tables 3 and 4 the D values have been calculated for each of the initial inoculum levels assumed. It may be seen that for each product the D values calculated from partial spoilage at more than one level of irradiation show good agreement. As would be expected when the assumed level of inoculum is decreased the calculated D value increases slightly. In Table 4 it is evident that D values calculated from partial spoilage produced by a 100 fold difference in initial inoculum show very good agreement.

TABLE 4

Calculation of radiation D values in whole kernel corn in brine for two different spore inoculum levels

Inoculum level per can	Dose megarad	Spoilage [1]	D value (megarad)		
			Log M = 9.3	Log M = 9.08	Log M = 8.6
10^8	2.70	9/20	0.32	0.33	0.35
			Log M = 7.3	Log M = 7.08	Log M = 6.6
10^6	1.86	12/20	0.30	0.31	0.34
	2.04	7/20	0.32	0.33	0.35
	2.32	1/20	0.32	0.33	0.35

[1] Number found/number tested

From the tables it is evident that there is no difference in the resistance of this suspension in chicken parts, steak, and whole kernel corn whether judged by "endpoint" comparison or by D value calculation. Resistance in pork loin may be slightly lower (10–12%) but at this stage of our experience it is difficult to say whether this percentage difference is reproducible and significant. Resistance in green beans cannot be compared with the other products based upon "endpoint" comparison since the inoculum level is different. On the basis of comparison of D values, however, the suspension appears significantly lower in resistance by 40–45%. This result is sufficiently striking to justify considerable additional study of this product.

The data have been presented primarily to illustrate a methodology for the determination of the resistance of spores of *Cl. botulinum* to ionizing radiation. The procedure, based upon high spore load inoculation and 20 can lots for each radiation level, provides data for both the "endpoint" type of evaluation and for D value calculations and therefore may satisfy the proponents of both approaches. There is no practical reason why the initial spore load should not be raised to one billion or higher per can for purposes of endpoint evaluation if desired. If one prefers the D value approach, it appears desirable—as often as possible—to determine the D value from initial spore loads at least 100 fold apart in order to further justify the procedure.

Results of the tests showing spoilage only by Type A strains where a mixture of Types A and B strains were used as inoculum, makes it appear inadvisable to use a mixture of strains in the evaluation of radiation resistance.

Although, in the work reported here, an incubation period of 12–15 months was used due to the circumstances under which it was conducted, an inspection of the spoilage data of this and other experiments suggests that the first level showing no spoilage could be examined after 6 months' storage.

SUMMARY

Procedures have been suggested for the evaluation of the resistance of spores of *Clostridium botulinum* in food products subjected to ionizing radiation and have been illustrated by representative data. The occurrence of nontoxic spoilage yielding either strongly toxic or questionably toxic cultures has been noted. Although a mixture of strains, 3 Type A and 2 Type B was used as inoculum, all survivors produced Type A toxin and it is suggested that the determination of radiation resistance with a suspension representing a mixture of strains is inadvisable. Approximately the same resistance of the inoculum was found in chicken parts, steak, and whole kernel corn; survival at 2.8—no survival at 3.0 megarad, radiation D value 0.33–0.35 megarad. In pork loin resistance appeared slightly lower but on the borderline of significance. Resistance of the suspension appeared definitely lower in green beans based upon a comparison of D values.

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A TOXICOLOGICAL STUDY OF BIPHENYL, A CITRUS FUNGISTAT

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Biphenyl is a fungistatic agent effective for preserving fresh citrus fruits. Although biphenyl has been used for this purpose for a number of years, both in this country and abroad, there is a paucity of published toxicity data to prove or disprove the safety of its use. The investigations of West *(4)*, and Deichman, Kitzmiller, Dierker, and Witherup *(1)* were not designed to secure data permitting evaluation of the health hazards that might arise from the repeated ingestion of minute amounts of biphenyl over long periods of time.

The most comprehensive toxicological study of biphenyl designed to supply information on the health hazards that might be associated with its use on citrus fruits was that undertaken at the Stanford Research Institute *(3)*[c]. This investigation showed that there were no significant differences between control monkeys and those which received 0.01% or 0.1% biphenyl in the diet for one year. Even on a dietary level of 1.0% biphenyl there were no gross or microscopic effects other than an apparent increase in liver weight. Thirty applications of biphenyl, over a period of 6 weeks, to the intact skin of rabbits produced no irritation or sensitivity, nor did microscopic examination of internal organs and skin show any abnormalities. Comparison of control rats with rats receiving 0.01%, 0.03%, and 0.1% biphenyl in the daily diet for 3 months revealed no significant differences in growth rates, food efficiency, blood urea nitrogen, organ weights, or microscopic examination of various tissues. Dietary levels of 0.01% and 0.1% biphenyl had no effect on reproduction in rats through 3 generations, but adverse effects were present at the 1.0% level.

The long term feeding experiments with rats at the Stanford Research Institute made use of dietary levels of 0.01%, 0.1% and 1.0% biphenyl for a two-year period. Unfortunately, this study failed to supply the necessary data for two reasons. A severe respiratory infection with a high mortality among the controls complicated the experiment. Secondly, rats receiving 0.1% and 1.0% biphenyl exhibited tubular dilation of the kidneys as did two of the control rats. Many of the remaining controls showed minimal tubular dilation such as that seen in the kidneys of rats receiving 0.01% biphenyl. The failure

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[c] The results of this investigation are summarized and commented upon with the kind permission of the Stanford Research Institute and the Crown Zellerbach Corporation.

to resolve the problem of determining a margin of safety between the maximum safe level of dietary biphenyl and the amount likely to be present in the human diet was recognized by the Stanford Research Institute. Their report states, "In view of the conflicting kidney pathology of the control rats and of the rats fed 0.01% diphenyl, it is impossible to ascertain the safe limits of diphenyl at this time."

Before concluding this brief summary of results obtained by the Stanford Research Institute, attention should be called to the factors which may have complicated interpretation of their results. Many strains of rats normally excrete urinary protein, and exhibit a certain degree of tubular dilatation of the kidneys. The extent of tubular dilatation may be influenced by the amount and kind of dietary protein as reported by Medlar and Blatherwick *(2)*. Finally, the respiratory infection may have resulted in toxins which contributed to the kidney damage.

EXPERIMENTAL

The following experiment was designed to secure the desired toxicological data lacking to date. Fifteen weanling albino rats of each sex were placed on each of the following levels of biphenyl added to the basal diet, 0.0, 0.001, 0.005, 0.01, 0.05, 0.10, 0.50, and 1.0%. These levels of dietary biphenyl were selected to permit: (1) repetition of the levels used by the Stanford Research Institute, (2) intermediate levels, and (3) low levels that might be anticipated to provide information regarding a margin of safety. The basal diet consisted of degerminated yellow corn meal 73%, crude casein 10%, linseed oil cake meal 10%, ground alfalfa 2%, bone ash 1.5%, sodium chloride 0.5%, and cod liver oil USP 3%. The rats were placed in well ventilated tiers of adequately sized cages, (16 by 16 in), 5 rats to a cage. Each cage was provided with feeding devices and water bottles which permitted free access to food and water at all times. During the period of growth the rats were weighed once a week and food consumption was determined. Following the period of active growth, the rats were weighed at 50-day intervals for the duration of the experiment. Hemoglobin determinations were made on male and female rats on the control diet and the 1.0% level of biphenyl every 100 days, on rats receiving 0.5% biphenyl at the end of 500, 600, and 700 days, and on rats receiving 0.1% biphenyl at the end of 500 and 700 days. Because of a slight impairment of growth and reduced food consumption noted on levels of 1.0 and 0.5% biphenyl, paired feeding experiments were conducted. A record was kept of all abnormal tissue growth noted grossly in both control and biphenyl-fed rats. At time of autopsy the weights of liver, kidneys, heart, and testes were determined. Hematoxylin-eosin stained sections of heart, lung, liver, kidney, adrenal, spleen, pancreas, stomach, intestine, bladder, thyroid, brain, pituitary, and gonads were prepared. Bone marrow smears of representative animals were prepared.

Studies on the effects, if any, of biphenyl on reproduction and survival of young were conducted as follows. Ten female rats and 5 males of weanling age were placed on the control diet for 60 days, and subsequently mated, one male to 2 female rats. An identical experiment employed a dietary level of 0.1% biphenyl. Nine female and 3 male rats were fed a dietary level of 0.5% biphenyl in a third experiment. All rats were kept on their respective diets until the young of all litters were weaned. In a second series of experiments 8 female and 4 male rats were placed on the control diet, 8 females and 4 males on 0.1%, and 9 females and 3 males on 0.5% dietary levels of biphenyl, all animals being 90 days of age and on their respective diets 11 days before being mated.

RESULTS

The percent hemoglobin found in male and female rats on the control basal diet and on dietary levels of 1.0, 0.5 and 0.1% biphenyl is given in Table 1. These data show that male and female rats receiving 1% biphenyl had lowered hemoglobin values after 300 and 400 days, respectively, and that 0.5% biphenyl resulted in lowered hemoglobin values in male and female rats at 500 and 600 days respectively. Since the paired

TABLE 1

Hemoglobin values ± S.E.* of rats on control and biphenyl (BP) diets

Days on diet	Males				Females			
	Control	1.0% BP	0.5% BP	0.1% BP	Control	1.0% BP	0.5% BP	0.1% BP
100	15.44 ± 0.35	15.57 ± 0.18			15.22 ± 0.33	13.87 ± 0.35		
200	15.11 ± 0.33	14.34 ± 0.61			15.47 ± 0.16	14.59 ± 0.39		
300	16.49 ± 0.31	14.86 ± 0.34			15.42 ± 0.25	14.96 ± 0.34		
400	16.04 ± 0.29	15.50 ± 0.24			15.22 ± 0.17	14.28 ± 0.22		
500	16.17 ± 0.31	14.27 ± 0.25	13.39 ± 0.80	14.87 ± 0.43	15.17 ± 0.36	13.27 ± 0.66	14.62 ± 0.32	15.28 ± 0.18
600	15.60 ± 0.38	13.55 ± 0.66	13.68 ± 0.59		16.22 ± 0.32	13.10 ± 1.13	12.50 ± 0.46	
700	14.99 ± 0.36	10.08 ± 0.77	12.13 ± 0.84	14.67 + 0.45	13.45 ± 0.85	9.45 ± 2.71	12.68 ± 0.36	13.33 ± 1.16

* S.E. = Standard error of the mean.

TABLE 2

Average body weight data for rats receiving biphenyl in their diets

Dietary concentration			Average body weight (grams)											
			Days (approximate)											
	%	ppm	0	50	100	150	200	250	300	350	450	550	650	730
Males														
	0.0	0	41	247	331	372	386	397	418	435	449	447	449	401
	0.001	10	41	228	309	357	374	390	406	411	437	449	444	423
	0.005	50	41	227	303	355	368	376	390	403	418	427	425	378
	0.01	100	41	236	311	355	370	383	405	423	431	443	433	428
	0.05	500	42	239	316	357	365	376	387	404	406	410	396	390
	0.1	1,000	42	239	322	352	366	367	378	395	406	413	407	393
	0.5	5,000	42	193	261	310	303	320	326	337	322	332	369	367
	1.0	10,000	42	143	199	223	248	252	260	272	285	286	228	—
Females														
	0.0	0	41	167	210	244	259	281	287	299	337	360	366	328
	0.001	10	41	170	217	253	267	282	292	298	321	340	345	375
	0.005	50	41	162	209	247	260	277	282	294	310	339	356	348
	0.01	100	41	169	208	244	260	278	283	293	323	348	397	357
	0.05	500	41	182	210	235	246	260	261	264	300	312	321	313
	0.1	1,000	40	162	207	235	252	257	252	253	304	337	343	332
	0.5	5,000	41	143	180	205	218	230	229	239	258	261	257	236
	1.0	10,000	41	131	152	169	177	187	187	195	202	195	188	—

feeding experiments with dietary levels of 1.0 and 0.5% biphenyl showed that inhibition of growth was due to decreased food intake, the lowered hemoglobin values may be due, in part, to the poorer nutrition.

Data on the average body weights of control rats and rats fed the various dosage levels of biphenyl are summarized in Table 2. These data show that the effects of dietary biphenyl on growth and final body weights are most pronounced on the 1.0 and 0.5% levels and are of doubtful significance on lower levels. Since records of food consumption revealed a decrease, paired feeding experiments with both male and female rats on levels of 1.0 and 0.5% biphenyl were conducted for 98 days to determine whether the decrease in growth could be accounted for by the lowered food intake. Thirty-eight male rats and 46 female rats were used in this experiment as indicated in Table 3, 6 rats of each sex being controls receiving food ad libitum. Table 3 summarizes the data on the average food consumption and the gain in weight. Growth rate data are shown graphically in Figures 1 and 2 for 1.0 and 0.5% levels of biphenyl, respectively. It is clearly evident that on both dosage levels both sexes receiving biphenyl grew at the

TABLE 3

Paired-feeding experiment (98 days); effect of biphenyl on body weight and food intake

Treatment	Males			Females		
	No. of rats	Body weight Grams ± S.E.*	Average food consumption grams	No. of rats	Body weight Grams ± S.E.*	Average food consumption grams
Controls fed ad libitum	6	232 ± 5.00	1313.3	6	150 ± 3.37	1143.8
0.5% BP	9	203 ± 4.58	1102.2	10	126 ± 2.91	941.0
Pair-fed Controls	9	199 ± 5.05	1101.0	10	123 ± 5.06	938.4
1.0% BP	7	172 ± 2.63	1012.7	10	113 ± 4.71	845.6
Pair-fed Controls	7	170 ± 6.32	1008.7	10	107 ± 3.27	846.1

* S.E. = Standard error of the mean.

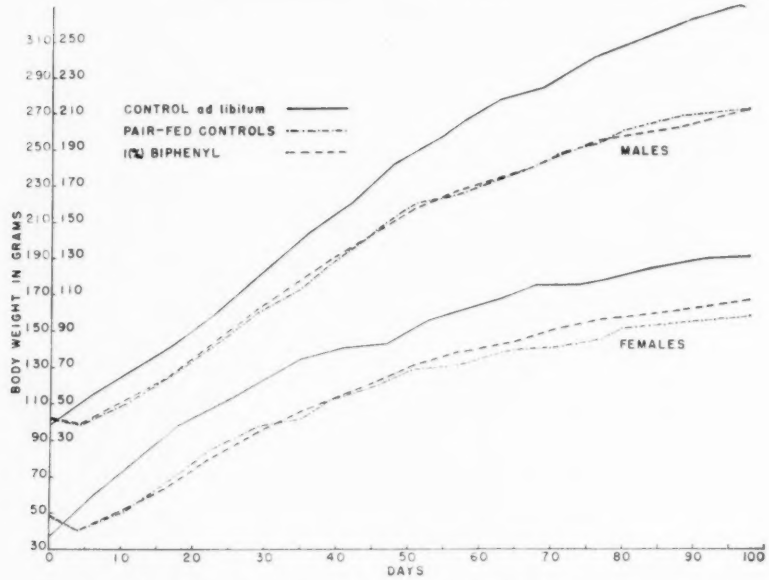

Figure 1. Growth rates of rats fed 1% biphenyl, pair-fed controls, and controls fed ad libitum.

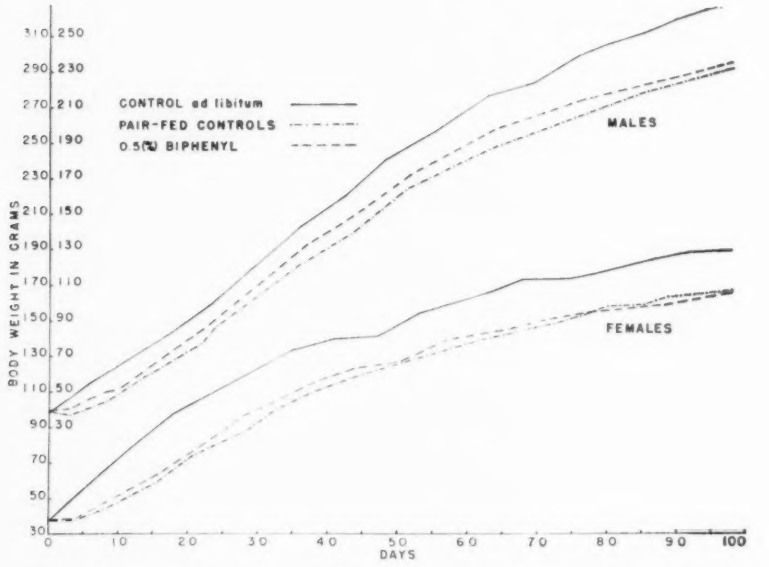

Figure 2. Growth rates of rats fed 0.5% biphenyl, pair-fed controls, and controls fed ad libitum.

same rate as the pair-fed controls, but more slowly than controls receiving food ad libitum. Moreover, the lower growth rate occurring on a diet containing 1.0% biphenyl as compared with 0.5% correlates with the food consumption data in Table 3. These observations suggest that growth retardation is due to a lowered food intake, possibly because of decreased palatability, rather than to a direct toxic action.

Table 4 records the number of survivors on the control and biphenyl diets at various periods throughout a 750-day period. Except on the 1.0 and 0.5% levels of biphenyl, there is no clear-cut evidence relating biphenyl intake to decreased longevity.

TABLE 4

Survival data for rats fed biphenyl for two years

	Dietary concentration of biphenyl		Days										
	%	ppm	0	50	100	150	200	250	300	350	450	550	650	750
			Number of survivors											
Males														
	0.0	0	15	15	14	14	14	14	14	13	12	12	10	9
	0.001	10	15	15	15	14	14	14	14	12	12	12	12	8
	0.005	50	15	15	15	15	15	15	15	14	14	14	13	10
	0.01	100	15	14	14	14	14	13	13	12	12	12	12	11
	0.05	500	15	15	14	14	14	14	14	14	14	14	14	13
	0.1	1,000	15	15	15	15	15	15	15	12	11	10	10	10
	0.5	5,000	15	15	14	14	14	14	14	11	9	9	5	2
	1.0	10,000	15	14	14	14	13	13	12	11	10	8	5	2
Females														
	0.0	0	15	15	15	15	15	15	15	15	15	13	12	9
	0.001	10	15	15	15	15	15	15	15	15	14	13	8	6
	0.005	50	15	13	13	12	12	12	12	11	10	10	6	5
	0.01	100	15	15	15	15	15	15	15	14	13	12	11	11
	0.05	500	15	15	15	15	15	15	15	12	11	11	9	5
	0.1	1,000	15	15	15	15	14	13	13	9	9	7	7	5
	0.5	5,000	15	15	15	14	14	14	13	11	11	9	6	5
	1.0	10,000	15	15	14	14	13	13	11	9	7	5	3	2

TABLE 5

Organ weights of male and female rats on diets containing biphenyl

Per cent biphenyl in diet	Days on diets	No. of rats	Body weights grams Mean ± S.E.*	Mean organ weights (grams/100 g body weight ± S.E.*			
				Liver	Kidneys	Heart	Testes
Males							
0.0	745	9	396 ± 24.6	2.89 ± 0.16	0.75 ± 0.02	0.32 ± 0.015	0.72 ± 0.03
0.001	744	8	424 ± 5.1	2.66 ± 0.06	0.70 ± 0.03	0.28 ± 0.008	0.62 ± 0.07
0.005	747	10	383 ± 19.8	2.84 ± 0.15	0.73 ± 0.02	0.30 ± 0.01	0.56 ± 0.06
0.01	752	11	394 ± 14.2	2.47 ± 0.07	0.72 ± 0.01	0.31 ± 0.008	0.67 ± 0.07
0.05	730	13	371 ± 15.8	3.03 ± 0.12	0.74 ± 0.02	0.31 ± 0.007	0.65 ± 0.06
0.1	746	10	366 ± 23.7	2.98 ± 0.19	0.83 ± 0.05	0.34 ± 0.012	0.60 ± 0.08
0.5	746	2	345	3.12	1.17	0.36	0.36
Females							
0.0	745	9	333 ± 9.4	3.11 ± 0.15	0.65 ± 0.01	0.33 ± 0.01	
0.001	744	6	369 ± 13.4	3.21 ± 0.17	0.62 ± 0.02	0.28 ± 0.07	
0.005	747	5	335 ± 16.6	2.81 ± 0.28	0.64 ± 0.02	0.31 ± 0.03	
0.01	752	11	341 ± 9.1	3.46 ± 0.74	0.62 ± 0.02	0.30 ± 0.01	
0.05	730	5	306 ± 12.5	3.51 ± 0.12	0.68 ± 0.02	0.31 ± 0.01	
0.1	746	5	327 ± 6.8	3.18 ± 0.10	0.65 ± 0.01	0.32 ± 0.01	
0.5	746	5	226 ± 25.8	4.52 ± 0.20	1.39 ± 0.14	0.46 ± 0.04	

* S.E. = Standard error of the mean.

Throughout the course of the experiment all rats were examined at time of weighing for gross evidence of tumors. No abnormalities were noted prior to 500 days on the experiment. Subsequently and at time of autopsy 28 animals showed gross evidence of tissue abnormalities. Most of these appeared to be mammary tumors and polyps. All abnormalities were in females except in 2 instances. In only one case, where the rat was incapacitated, was an animal sacrificed prior to termination of the experiment.

Data for body weights and weights of liver, kidneys, heart and testes of rats on control and biphenyl diets are summarized in Table 5. In summary, there appears to be no significant variation except in the case of female rats receiving 0.5% biphenyl where the body weight decreased and weights of liver and kidneys increased.

As shown in Table 6 dietary levels of 0.1 and 0.5% biphenyl had no significant effect on reproduction. These results are in accord with those of the Stanford Research Institute.

TABLE 6

Summary on reproduction data

Exptl. series	Diet	No. of rats casting litters	Total born	Range of litter size
1[1]	Control	9 out of 10	59	3– 9
	0.1% Biphenyl	10 out of 10	67	2–10
	0.5% Biphenyl	8 out of 9	53	3– 9
2[2]	Control	8 out of 8	64	5–13
	0.1% Biphenyl	6 out of 8	63	3–10
	0.5% Biphenyl	8 out of 9	48	3– 9

[1] Weanling rats on diets 60 days before mating.
[2] Ninety-day-old rats on diets 11 days before mating.

HISTOPATHOLOGICAL FINDINGS

The only histopathological change clearly related to the ingestion of biphenyl occurred in the kidneys. The kidneys of all male and female rats on dietary levels of 0.5 or 1.0% biphenyl had prominent irregular scarring, lymphocytic infiltration, tubular atrophy, and patchy tubular dilation to the point of cyst formation. Hemorrhage was present in some of the dilated tubules as well as in the renal pelvis in some instances. Calculi with basophilic staining foci were frequent in the renal pelvis and similar smaller deposits of precipitated material were sometimes seen in the kidney substance. Certain of the dilated tubules contained a good many polymorphonuclear leucocytes and small fragments of nuclear material. Hydronephrosis was common and in several instances there was metaplasia of the epithelium of the renal pelvis to the squamous cell type, but this did not appear neoplastic.

The kidneys of the female rats on doses of 0.1% or less exhibited no changes that were clearly different from occasional small scars and focally dilated tubules which were present in the control animals.

In the kidneys from the male animals at all dose levels including the controls, scars and dilated tubules were distinctly more numerous and some degree of hydronephrosis more prominent than in the females. This corresponded to the gross demonstration of deposited material in the renal pelvis or bladder in male animals only, except at the 0.5 and 1.0% levels where it was present in female rats. Most of the kidneys from male rats which received 0.1% or 0.05% biphenyl were not distinguishable from the controls, although in two of these animals there were masses of partly disintegrated blood in the renal pelvis and in two others there were small basophilic concretions in

the medullary portions of the kidneys. Blood was present in the renal pelvis also in one animal from each of the other treated groups (0.01, 0.005 and 0.001%). These deposits were sometimes associated with hydronephrosis. Hydronephrosis was present also in several kidneys from the different groups of male animals (including controls) in which pelvic hemorrhage or concretions were not demonstrable. Some of these animals, as well as some others without observed hydronephrosis, presented a protein coagulum in the bladder. This was present in several of the control animals and was clearly unrelated to the treatment. In one control kidney there was a small amount of old blood in the pelvis.

Comparison of the kidneys from the various groups of animals indicated that with doses of 0.1% biphenyl or less there was no distinct difference from the controls and no evidence of a relationship between the presence of kidney lesions and the consumption of biphenyl.

No other organ changes could be related to biphenyl ingestion. There was irregular atrophy of testes and ovaries in the different groups of animals, but even with the larger doses of biphenyl some animals exhibited relatively little alteration of the gonads. Occasional inflammation of the respiratory tract was not related to the treatment. Focal scarring of the myocardium, occurring mostly in males, was no greater in animals which received large doses of biphenyl than it was in control animals. No evidence of liver injury by the compound was found. Study of blood and bone marrow from a number of the animals yielded no evidence of injury to blood-forming structures. Some variation in cellularity of the marrow was unrelated to the treatment.

TABLE 7

Incidence and location of tumors in control and biphenyl-fed rats

	Breast	Hypophysis	Adrenal	Uterus	Lung	Bladder	Other
Male controls		1/7	2/7			2/9	
Male 0.001%		1/8	1/6			2/8	1/9
Male 0.005%		2/6					
Male 0.01%		1/8			1/11	1/9	
Male 0.05%		1/12	1/13		2/13		
Male 0.1%		1/8	1/9		1/9	1/9	
Male 0.5%						1/2	
Male 1.0%						1/2	1/2
Female controls	3/9	2/7		2/9		1/9	
Female 0.001%	3/6	3/5		1/6	1/6		
Female 0.005%	2/6			1/6			
Female 0.01%	4/11	3/10		4/11			
Female 0.05%	1/5	1/3	2/5		2/5		
Female 0.1%				2/5	1/5		
Female 0.5%	1/5	1/3	2/5				
Female 1.0%							

Table 7 shows the incidence of tumors in various locations of control and experimental rats. There was no apparent relation between the occurrence of these lesions and the character of the treatment. No tumors were found in any of the livers of control or experimental rats. The lesions classified as bladder tumors varied from local zones of papillary proliferation of the lining epithelium to distinct protruding tumors, but none showed clear-cut malignant

changes. All tumors of the breast, hypophysis, and adrenal were benign. Five of the 8 lung tumors were carcinomas. However, since no lung tumors were found on the two highest dosage levels of biphenyl and the 2 lesions on the 0.1% level were benign, it seems unlikely that the 5 instances of malignancy on the lower dosages are attributable to the biphenyl. Only one of the 10 uterine tumors was malignant and it occurred on the 0.1% level of biphenyl. One carcinoma occurred in the pancreas of a control female rat, and one unclassified proliferative lesion was present in a piece of fatty tissue of a male rat fed 1.0% biphenyl.

In a duplicate long-term feeding experiment with rats Purina Laboratory Chow [d] was used as the basal diet. The results were comparable with those reported above as regards growth, longevity, histopathological changes, and dietary levels of biphenyl responsible for kidney lesions.

CONCLUSIONS

The inhibition of growth of both male and female rats on dietary levels of 0.5 and 1.0% biphenyl is due to decreased food consumption.

Hemoglobin values of male and female rats on 1% level of biphenyl were decreased at 300 and 400 days respectively, and at 500 and 600 days, respectively, on 0.5% biphenyl. This effect may be due, in part at least, to a decrease in food consumption.

The only lesions definitely attributable to biphenyl occurred in the kidney of male and female rats fed 0.5 and 1.0% biphenyl. These kidney lesions are reversible when the animals are returned to the control diet as will be reported in a separate publication.

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[d] Mention of this product does not imply endorsement over others of similar nature.

FACTORS IN EGG WHITE WHICH CONTROL GROWTH OF BACTERIA

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Previous studies from this *(10)* and other laboratories *(12, 18)* have indicated that the role of both the shell and shell membranes in the protection of eggs from bacterial spoilage is of a mechanical nature. Although the shell, complete with the bloom, is able to restrict somewhat the entry of microorganisms, penetration of the membranes presents the bacteria with a more serious barrier. As shown previously, several days are usually necessary for the bacteria to overcome this obstruction *(10)*. The mechanical nature of the protective mechanism was indicated by the finding that all bacteria tested, which included 50 strains of 8 different genera, were able to penetrate the shell membranes without significant decomposition of the membrane material.

After piercing the shell and membranes the bacteria face a new and more formidable obstacle; namely, the albumen. The latter is known to contain several factors which inhibit the growth of bacteria: high pH *(17)*, lysozyme *(6)*, avidin *(3)*, ovomucoid *(2)*, conalbumin *(15)*, and the presence of only native proteins *(14)*.

There can be no doubt that lysozyme-sensitive microorganisms (primarily Gram-positive bacteria) do not multiply or even survive in egg white. Similarly, those bacteria which require biotin for growth would be prevented from multiplying in egg white by the high concentration of avidin, the biotin-binding egg white protein. Ovomucoid, the anti-trypsin factor has been mentioned as affecting growth *(2)*. Conalbumin, the iron-binding protein, prevents growth by making iron unavailable to microorganisms. Finally, the high pH attained by egg white shortly after the egg is laid has been considered to inhibit microbial growth.

The relative importance, however, of each of these factors in the prevention of microbial egg spoilage has not been determined and is the object of the present investigations.

MATERIALS AND METHODS

Bacteria. The bacteria were those previously described *(10)*.

Egg white. Sterile egg white was prepared in the following manner: Several day-old eggs were immersed in 70% alcohol for 5 min. The eggs were removed, the excess alcohol allowed to drain and the residual alcohol removed by flaming the shell. This procedure was effective in destroying the microbial population on the shell. The egg was cracked and the contents were separated on a sterile commercial tray and separator. The egg white was collected in a sterile beaker and blended in a sterile blender jar at 50 volts for 1 min. Incorporation of air into the white during blending was prevented by submerging the base of a sterile Erlenmeyer flask into the top layer of the egg white. After blending, the egg white was stored in sterile flasks at 4° C and used within 1 or 2 days.

[a] A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

Sterility of these preparations was checked by inoculation of 5 ml of egg white into 100 ml of sterile trypticase-soy broth and incubation at 28° C for several days.

Sterile supplements were introduced into the egg white while it was being blended. This minimized the danger of denaturation of the egg white proteins by preventing any localization of a high concentration of acid, alkali, or iron salts.

Supplements. (a) 1*N* HCl and 1*N* NaOH used to adjust the egg white to the desired pH were sterilized at 15 lb steam pressure for 15 min.

(b) $FeSO_4$—aqueous solutions containing 2.0 mg Fe^{2+} per ml (9.95 mg $FeSO_4 \cdot 7\ H_2O$ per ml) were sterilized by filtration through ultrafine sintered glass funnels.

Inoculum. Bacteria from a trypticase agar slant were transferred to nutrient broth supplemented with 0.5% yeast extract. After one day's incubation at 28° C, the culture was either centrifuged and washed 2 times with distilled water before use, or was diluted directly in sterile distilled water to give the desired level of inoculum. In the latter case, dilutions were always one to 10,000 or greater, so that no significant carry-over of nutrients would occur.

Plate counts. Changes in bacterial numbers were determined by spread plating 0.1 ml of the appropriate dilution on trypticase soy agar plates. Colonies were counted after incubation of the plates at 28° C for 2 days.

RESULTS

Ability of bacteria to multiply in egg white. The fate of bacteria in contact with albumen was investigated. Bacteria *(10)*, originally isolated from spoiled eggs, were inoculated into egg white in sterile screw-top test tubes. Initial populations ranged from 100 to 4,000 bacteria per ml. The inoculated egg white was incubated at 28° C. After one week's incubation only 27 of the 61 strains of bacteria tested had recoverable numbers, and only 2 strains had multiplied to any extent. After 2 weeks, 15 strains had viable bacteria present, with only 7 reaching populations greater than 10^6 per ml.

The inability of most of these bacteria to survive and multiply in undiluted egg white presented a situation which could be used to determine the relative effectiveness of the several substances and conditions known to exist in egg white in inhibiting growth of these bacteria.

Lysozyme. Since the bacteria used in this study are Gram-negative, one would expect them to be resistant to the lytic action of lysozyme. This was indicated by the slow death rate of representative organisms inoculated into egg white and incubated at 28° C (Table 1). In contrast, when *B. subtilis* NRRL B-1471 (which is Gram-

TABLE 1

Growth of bacteria in egg white

Organism	Population (bacteria/ml) at			
	0 hours	20 hours	92 hours	216 hours
Paracolobactrum aerogenoides 72c	2.3×10^7	1.2×10^9	1×10^9	1×10^9
	2.3×10^6	1.9×10^8	5.6×10^8	1.1×10^9
	2.3×10^5	1.0×10^3	1.2×10^4	3
	2.3×10	2.0×10	0	0
Alcaligenes bookeri 22b	6.2×10^7	5.3×10^7	1.8×10^8	2.2×10^8
	6.2×10^6	5.6×10^6	1.3×10^8	4.6×10^8
	6.2×10^5	1.6×10^5	3.8×10^5	1.0×10^7
	6.2×10	2.4×10	2.0×10	2.4×10
Pseudomonas fluorescens 13c	1.2×10^7	5.4×10^7	5.2×10^7	2.0×10^3
	1.2×10^6	4.9×10^6	<200	<200
	1.2×10^5	1.4×10^3	<200	0
	1.2×10	4.3×10	0	0
Bacillus subtilis NRRL B-1471	0[1]			

[1] Inoculated to give 10^6 bacteria/ml. Zero time count made immediately after mixing.

positive and lysozyme-sensitive) was introduced into egg white to give initial populations of 10^6 cells per ml, no viable bacteria could be recovered from the egg white immediately after mixing.

pH. The initial survey experiments were conducted with egg white from eggs several days old. It is well known that the pH of egg white at the time of laying varies between 7.6 and 7.8. Immediately thereafter the egg loses CO_2, and after a few days at room temperature the white reaches a pH of 9.1 to 9.3 *(16)*. Sharp and Whitaker *(17)* have shown that this pH can inhibit growth of several species of bacteria. Therefore the growth of bacteria in egg white at its pH under storage conditions (9.1 to 9.3) was compared with the growth in egg white adjusted to a pH approximating that in the egg at laying time. Results of the study are given in Table 2.

TABLE 2

Effect of pH on growth of bacteria in egg white

Organism	Group No.	Initial pH	Population (bacteria/ml) at			
			0 hours	66 hours	184 hours	232 hours
Pseudomonas fluorescens 7	1	9.1	1.1×10^5	$<10^3$		$<10^3$
		7.9	1.1×10^5	7×10^5	3.1×10^7	1.3×10^8
Proteus vulgaris 53a	2	9.1	1.6×10^5	9.6×10^4		3×10^5
		7.9	1.6×10^5	2.3×10^6	1.1×10^8	2.0×10^8
Alcaligenes faecalis 18b	3	9.1	1.4×10^5	5×10^3		2.1×10^4
		7.9	1.4×10^5	4.9×10^4	8.5×10^4	1.9×10^8
Alcaligenes faecalis 29c	4	9.1	4.8×10^4	2.5×10^4		2.4×10^6
		7.9	4.8×10^4	1.1×10^7		1×10^7
Alcaligenes bookeri 22b	5	9.1	1.5×10^5	5×10^3		$>2 \times 10^6$
		7.9	1.5×10^5	2.8×10^6	1.1×10^8	1.9×10^7

Bacteria classified in group 1 are those able to multiply in egg white at pH 7.9 but do not survive at pH 9.1: group 2 contains bacteria which survive but do not multiply at pH 7.9 and die at a slow rate at pH 9.1; group 3 contains those which survive but do not multiply at either pH 9.1 or 7.9; group 4 contains those surviving at pH 9.1 and growing at pH 7.9; and group 5 are those able to grow at both pH 7.9 and 9.1.

Other bacteria tested could be classified as follows: Group 1, *Pseudomonas ovalis* 1, *Proteus melanovogenes* 54a, *Paracolobactrum intermedium* (a) 56a, *Paracolobactrum aerogenoides* 72c, *Aerobacter cloacae* 24a, *Alcaligenes recti* 9b, *Alcaligenes bookeri* 80, and *Proteus vulgaris* 65a. Group 2, *Paracolobactrum intermedium* (b) 56c, and *Escherichia freundii* 71c. Group 3, an unidentified strain of *Flavobacter* 22c. Group 4, *Alcaligenes faecalis* 29c was the only bacterium classified in this group. *Alcaligenes bookeri* 22b (group 5) was the only bacterium found to grow extensively in egg white at both pH 7.9 and pH 9.1.

Several strains of bacteria that are unable to grow or survive at pH 9.1 can do so if the pH is adjusted to 7.9, but there are several species which are unable to grow at this more favorable pH. There must be, therefore, some other factor still operating at this lower pH.

Conalbumin. This egg white protein is known to function by combining with iron to make it unavailable to microorganisms *(15)*. To determine the role that conalbumin plays in the protection of eggs from spoilage organisms, the growth of bacteria in egg white whose conalbumin had been saturated with iron was determined. Sterile egg white was prepared as previously described. The conalbumin was saturated by adding 20 mg of Fe^{2+} (as a sterile solution of $FeSO_4 \cdot 7\,H_2O$) per liter of egg white. A portion of this egg white was adjusted to pH 7.9 with sterile 1*N* HCl. After placing 5-ml aliquots in sterile screw-top test tubes, the contents of the tubes were inoculated with the bacteria under test and incubated at 28° C. Changes in bacterial

TABLE 3

Effect of added iron on growth of bacteria in egg white

Organism	Initial pH	Supplement	Population (bacteria/ml) at	
			0 hours	66 hours
Pseudomonas fluorescens 7	9.1	none	1.1×10^6	10^3
	7.9	none	1.1×10^6	7×10^5
	9.1	20γ Fe^{2+}/ml	1.1×10^6	4.8×10^8
	7.9	20γ Fe^{2+}/ml	1.1×10^6	1.7×10^8
Proteus vulgaris 53a	9.1	none	1.6×10^6	3×10^3
	7.9	none	1.6×10^6	2.0×10^5
	9.1	20γ Fe^{2+}/ml	1.6×10^6	2.2×10^9
	7.9	20γ Fe^{2+}/ml	1.6×10^6	1.6×10^8

numbers were determined after 66 hours' incubation. Results of the experiment are given in Table 3. It can be seen that addition of sufficient iron to saturate all the conalbumin in the egg white allowed the bacteria to develop to populations in excess of 10^8/ml. All 17 strains of bacteria tested behaved similarly.

Effect of inoculum size on growth of bacteria in egg white supplemented with iron. Present studies have shown that the numbers in the initial population were important in determining the ability of some bacteria to develop in unsupplemented egg white (Table 1). It therefore seemed desirable to study the effect of initial population on development of bacteria in egg white whose conalbumin was saturated with iron.

Sterile egg white was supplemented with 20 mg of Fe^{2+}/liter. Nine-ml aliquots were dispensed in 5 sterile screw-top test tubes; a sixth tube contained unsupplemented egg white. Inoculum was added to tubes 1 and 6 to give initial populations of approximately 10^6 bacteria/ml. Tube 1 was thoroughly mixed and 1 ml of the suspension was used to inoculate tube 2. This procedure was repeated so that tube 5 contained approximately 10 to 50 bacteria/ml. After incubation at 28° C for 48 and 192 hours, changes in population were determined by spread-plating appropriate dilutions. Results with a typical bacterium are given in Table 4.

Eleven bacterial strains were so tested and all gave essentially the same results.

Effect of graded levels of iron on growth of bacteria in egg white. We have shown that all Gram-negative bacteria tested will grow in egg white supplemented with sufficient iron to saturate the conalbumin. They will develop at both pH 7.9 and 9.1 under these conditions. Some bacteria also have the ability to grow in unsupplemented white at pH 9.1, while still more can do so if the white is first acidified to pH 7.9. This indicates that certain bacteria may be able to meet their iron requirements more readily than others. The following experiment was conducted to test this hypothesis.

Sterile egg white was divided into 4 aliquots. One was not supplemented; the second was supplemented with a sterile solution of $FeSO_4$ to give a concentration of 1.0 mg Fe^{2+}/liter; the third was supplemented so that approximately 35% of the conalbumin was saturated with iron (7 mg Fe^{3+}/liter egg white); and the fourth was supplemented with iron in excess of that needed to saturate all the conalbumin (20 mg

TABLE 4

Effect of initial bacterial numbers on their growth in egg white supplemented with iron

Organism	Supplement	Population (bacteria/ml) at		
		0 hours	48 hours	192 hours
Aerobacter cloacae 24a	none	5×10^4	1.1×10^9	2.1×10^9
	20γ Fe^{2+}/ml	5×10^4	1.5×10^9	
	20γ Fe^{2+}/ml	5×10^3	2.0×10^9	
	20γ Fe^{2+}/ml	5×10^2	2.0×10^9	
	20γ Fe^{2+}/ml	5×10	4.7×10^8	
	20γ Fe^{2+}/ml	5	4.1×10^7	1.5×10^8

Fe^{2+}/liter egg white). The various treatments were dispensed into sterile screw-top test tubes, inoculated to give counts of approximately 10^6 bacteria per ml, and incubated at 28° C. Populations were determined after 72 and 168 hours of incubation. Typical results of this experiment are given in Table 5.

TABLE 5

Effect of graded levels of iron on growth of bacteria in egg white

Organism	Iron added to egg white (mg Fe^{2+}/liter)	Population (bacteria/ml) at		
		0 hours	72 hours	168 hours
Pseudomonas fluorescens 7	none	1.1×10^6	1.0×10^9	2.0×10^9
	1.0		5.4×10^9	2.2×10^9
	7.0			2.8×10^7
	20.0		2.3×10^7	1.7×10^8
Proteus melanovogenes 54a	none	0.7×10^6	3.3×10^6	5×10^9
	1.0		$>>10^9$	4.1×10^8
	7.0			2.0×10^9
	20.0		7.8×10^8	6.7×10^9
Proteus vulgaris 53a	none	1.3×10^6	2.1×10^4	2.2×10^4
	1.0		1.2×10^5	1.0×10^4
	7.0			1.0×10^4
	20.0		2.7×10^9	2.3×10^6

Proteus melenovogenes 54a is able to satisfy its iron requirement in egg white supplemented with only 1.0 mg of iron/liter. On the other hand *Proteus vulgaris* 53a is not able to grow in egg white unless it contains iron in excess of that necessary to saturate all the conalbumin present. Other strains of bacteria showed some capability of obtaining iron from egg white whose conalbumin is only partially saturated with this essential nutrient.

Effect of high pH on growth of bacteria in iron-supplemented egg white. Previous experiments have shown that all bacteria tested multiplied extensively in egg white at pH 9.1, provided the conalbumin were saturated with iron. Egg white under certain storage conditions may reach pH values as high as 9.6 *(16)*. It was of interest to determine whether egg spoilage bacteria could tolerate this pH and multiply readily if the conalbumin were saturated with iron.

Sterile egg white, supplemented with 20 mg Fe^{2+}/liter, was adjusted to pH 9.1, 9.2, 9.35, 9.5, and 9.6 with sterile 1*N* NaOH. Aliquots of 5 ml were dispensed in sterile screw-top test tubes. Unsupplemented egg white at pH 9.1 served as a control. The various test media were inoculated to give initial populations of approximately 10^4 bacteria/ml and incubated at 28° C. Changes in population density were determined after several days' incubation. Typical results are given in Table 6.

TABLE 6

Effect of pH on growth of bacteria in egg white supplemented with excess iron

Organism	Egg white [1]	Egg white supplemented with 20 mg Fe^{2+} (as $FeSO_4 \cdot 7H_2O$)/liter [1]				
	pH 9.1	pH 9.1	pH 9.2	pH 9.35	pH 9.5	pH 9.6
Ps. ovalis 1	1+	4+	4+	4+	4+	3+
Ps. fluorescens 7	0	4+	3+	2+	0	0
Alcaligenes bookeri	0	4+	4+	4+	4+	4+
Aerobacter cloacae	0	4+	0	0	0	0

[1] Initial population: 10^4 bacteria/ml

4+ $>10^8$ bacteria/ml
3+ $>10^7$ and $<10^8$ bacteria/ml
2+ $>10^6$ and $<10^7$ bacteria/ml
1+ $>10^5$ and $<10^6$ bacteria/ml
0 $<$ initial population

These results show that most of the bacterial strains tested can tolerate pH's in excess of those encountered under normal storage conditions. In fact, more than 50% of the strains tested grew readily at pH 9.5 and 4 of 10 grew extensively at the highest pH tested (pH 9.6) in the presence of iron.

Ovomucoid. This egg white protein has been mentioned as one of the antibacterial substances in egg white. Its known biological activity is its ability to combine with and inhibit the proteolytic enzyme, trypsin *(13)*. To determine whether ovomucoid affected the growth rate in any way, its effect on growth of several bacteria in a nutritious medium was determined.

The basal medium consisted of the following ingredients: nutrient broth, 8 g; yeast extract, 5.0 g; and distilled water to give 800 ml of 5/4 strength medium. The medium, 8 ml/test tube, was sterilized at 15 lb steam pressure for 15 min. After cooling, 2 ml of sterile distilled H_2O were added to one set of tubes and 2 ml of sterile ovomucoid solution [sterilized by filtration] were added to a duplicate set of tubes.

The contents of the tubes were inoculated with a drop of 19-hour cultures grown on the basal medium at 28° C and were incubated at this temperature. The optical density of the cultures at 610 mmc was determined hourly with the uninoculated control set at a reading of 0.05.

The growth rate and duration of the lag period of the bacteria tested were unaffected by ovomucoid. The bacteria tested included *Ps. fluorescens* 7, *Ps. ovalis* 1, *Alcaligenes bookeri* 22b, *Proteus vulgaris* 53a, *Proteus melanovogenes* 54a, *Aerobacter cloacae* 24a, *Proteus vulgaris* 65a, *Escherichia freundii* 71c, and *Paracolobactrum aerogenoides* 72c.

The anti-tryptic activity of ovomucoid was determined on the uninoculated control by modification of Anson's hemoglobin assay method for trypsin *(1)*.

Results of this assay showed that 0.2 ml of the test solution inhibited completely the proteolytic activity of 100 mcg of trypsin.

DISCUSSION

Various factors have been implicated as playing a role in protecting the egg against bacterial spoilage. The bloom, the shell, and the egg shell membranes are mechanical barriers against penetration to the egg contents. After the microorganism penetrates these defenses, it comes in contact with the albumen which contains several antibacterial substances. There is no doubt that lysozyme protects the egg contents by its lytic action on lysozyme-sensitive bacteria. This conclusion is substantiated by results of studies which showed that only Gram-negative bacteria were isolated from spoiled eggs *(7)*.

Similarly, microorganisms having a strict requirement for biotin would be unable to develop in egg white. The presence of avidin, the biotin-binding protein, would make this growth factor unavailable to bacteria.

Of the remaining protective factors, the most important role in controlling microbial growth in egg white is played by conalbumin. As shown by results of studies with iron, all bacteria tested developed to dense populations in egg white, provided the conalbumin were saturated with respect to iron. Even levels of inoculum, 10 to 50 bacteria per ml, were able to multiply rapidly and reach populations in excess of 10^8 bacteria per ml.

That high pH *per se* is not inhibitory is shown by the ability of many of the bacteria to grow in egg white at a pH more alkaline than normally reached by eggs under storage, provided the inhibitory action of the conalbumin were neutralized by addition of iron. All bacteria tested, except *Aerobacter cloacae* 24a, were able to grow at pH 9.4; 4 strains reached populations of greater than 10^7 bacteria per ml at pH 9.6.

The effect of pH on growth of bacteria in unsupplemented egg white may be explained by the action of hydrogen ion concentration on the availability

of iron. The average iron content of egg white has been reported as approximately 0.30 mg per 1000 g *(11)*. This amount of iron, if it were all available to bacteria, would be adequate to satisfy the iron requirement of highly aerobic microorganisms *(4)*. However, in egg white, iron exists in the tightly bound form as the iron-conalbumin chelate and is therefore not readily available to microorganisms.

In formation of the iron-conalbumin coordination compound, Warner and Weber *(19)* have shown that 3 protons are displaced from the iron-binding protein. There is, therefore, a competition between iron and hydrogen ion for the binding sites on the protein. One would expect, therefore, that an increase in hydrogen ion concentration would result in displacement of iron from the iron chelate making this trace element available to bacteria. In fact, below pH 5.5, the color of the coordination compound decreases and finally disappears completely below pH 4 *(19)*. This shift in equilibrium must also occur, although to a lesser extent, when the pH is decreased from 9.0 to 8.0. This effect of pH on the inhibitory action of conalbumin was nicely shown by Feeney and Nagy *(5)*.

Results in Tables 2 and 5 indicate that all bacteria do not have the same capacity to avail themselves of iron that is bound by conalbumin. Some are able to obtain sufficient iron if the egg white is first adjusted to pH 7.9. Others cannot utilize iron naturally present in egg white even at this lower pH.

Similarly when one supplements egg white with 1.0 mg Fe^{2+} per liter, which saturates only 5% of the conalbumin, *Proteus melanovogenes* 54a and *Proteus vulgaris* 65a are able to obtain sufficient iron to reach populations greater than 10^8 bacteria per ml (Table 5). Others are able only partially to satisfy their iron requirements and therefore cannot grow as extensively. In contrast there are some which cannot utilize any more iron under these conditions than in unsupplemented egg white, i.e., *Proteus vulgaris* 53a.

By supplementing egg white with 7.0 mg. of Fe^{2+} per liter (which saturates one-third of the conalbumin) several strains of bacteria grew to populations 100 times that reached in unsupplemented white.

One must conclude that microorganisms differ in ability to satisfy their iron requirements in the presence of excess conalbumin. It has been shown that some microorganisms secrete iron-binding agents when grown on media containing insufficient iron to meet their needs *(9)*. The stability constants of the iron chelates of two such compounds are of similar order of magnitude as that of the iron-conalbumin compound *(8)*. Since the chelating compounds elaborated by various microorganisms were not identical, their ability to bind iron would also differ. If one postulates that the bacteria we have used in this study also secrete different iron-binding compounds, the difference in ability of these bacteria to overcome the inhibitory action of conalbumin is understandable. Preliminary experiments have shown that several strains of spoilage bacteria secrete such compounds when grown in media containing low concentrations of iron.

SUMMARY AND CONCLUSIONS

Conalbumin, one of the inhibitory substances present in egg white, plays a major role in preventing growth of Gram-negative spoilage bacteria. The inhibition of growth in egg white is completely reversed by saturating the conalbumin with iron.

The high pH found in egg white shortly after the egg is laid did not inhibit growth of any of the bacteria tested when supplemented with sufficient iron to saturate the conalbumin. The inhibitory action of pH in unsupplemented egg white can be explained by its effect on the dissociation of the iron-conalbumin coordination compound. The stability of the latter increases as pH increases and thereby iron becomes less available to the microorganisms.

Ovomucoid has no effect on rate or extent of growth of Gram-negative bacteria.

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HEAT RESISTANCE AND GROWTH CHARACTERISTICS OF MICROORGANISMS ISOLATED FROM SEMI-PERISHABLE CANNED HAMS

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Considerable interest has developed in recent years in the food industry to determine the types and numbers of microorganisms present on food products as they are being processed. Also of interest are the types of organisms present after processing in the case of non-sterile items. This study concerns the natural flora present on hams for canning and its resistance to heat. The ham was selected because a large tonnage of this meat product is sold as a non-sterile or semi-perishable canned item.

Ingram and Barnes *(7)*, Barnes and Ingram *(2)* and Buttiaux *(4)* studied the problem of *Streptococci* in pasteurized canned hams. In the two former papers it was concluded that *Streptococci* isolated from canned hams and from the gut of the pig belonged to the same species *Strep. faecium* (Orla-Jensen); true *Strep. faecalis* organisms were not found. Ingram and Hobbs *(8)* made a routine examination of 175 canned hams. Both vegetative and spore forms were found. Jepsen *(9)* made a survey in 1947 of the microbial contamination of pork acquired in the Danish slaughterhouse. He examined 239 strains of non-spore forming bacteria and 67 strains of spore forming bacteria isolated from pig carcasses during slaughter.

MATERIALS AND METHODS

Samples of ham were removed twice a week from the Ottumwa plant canning line during the period September 4, 1957, to November 26, 1958. Each sample represented approximately 50 g of lean meat from each of 10 hams. The individual sample was the lean area above the aitch bone separated from the rest of the ham during boning. This surface sample was taken from a sampling area previously determined to be the most highly contaminated portion of the ham.

Total counts and pasteurized counts were made on Tryptone, Dextrose, Yeast Extract Agar with brom-cresol purple dye as an indicator. Duplicate plates were incubated for 48 hr at 35° C. Per cent salt in the ham was determined by a slight modification of the AOAC Method *(10)*. Per cent moisture was determined by drying a 10-g sample with the Brabender rapid moisture tester. Per cent brine was calculated using the following equation:

$$\% \text{ Brine} = \frac{\% \text{ salt}}{\% \text{ moisture} + \% \text{ salt}} \quad (100)$$

Heat resistance of the organisms was determined by aseptically grinding the meat and filling into a sterile Alemite gun. At least 10 clean, sterile, 10 x 75 mm chemical test tubes were then filled with approximately 1 g of meat each. Tubes were sealed in a blast lamp. A 5-min preliminary process in an oil bath at 175° F was used on tubes processed at F_0 (minutes at 250° F) values of 0.05, 0.2, 0.6, 1.0 and 3.0. No preliminary heating was used on tubes processed at F^1 (minutes at 150° F) values of 1 to 900. Processing was done in an oil bath controlled to plus or minus 0.1° C and upon removal the tubes were immediately transferred to cooling water at 70° F. Four tubes used at each heating level were subcultured into glucose brain broth. Remaining tubes were incubated at 32–36° C. Detailed explanation of our thermal processing techniques were

described by Stumbo, Gross, and Vinton *(11)*. The method of Gross and Schaub *(5)* was used to calculate the pasteurization processes.

EXPERIMENTAL DATA AND RESULTS

Table 1 summarizes the basic information obtained from analyzing ham samples taken twice a week for a 58-week period. The data represent over 1000 hams. Since samples were removed from the most contaminated area of each ham, it should be emphasized that counts are much higher than those from our regular plant production of canned hams. This bacterial load represents a maximum number of organisms present on the hams.

TABLE 1

A summary of the bacteriological condition of ham surface samples over a 58-week period

	Average
Total count	210,000 organisms per gram
Pasteurized count[1]	451 organisms per gram
Breakdown of pasteurized counts	
Samples — Organisms per gram	
25 — less than 10	
45 — 10–100	
41 — over 100	
Temperature in can	9.4° C (49° F)
Per cent salt	2.8%
Per cent moisture	62.0%
Per cent brine	4.3%

[1] This represents the average plate count after processing the ham sample to an internal temperature of 150° F.

TABLE 2

Heat resistance of organisms determined by subculturing immediately after processing

Processing level F_o units (Min at 250° F)	Average per cent positive samples (over 100 samples, each sample from 10 hams)
0.05	56.8
0.2	42.7
0.6	16.2
1.0	19.7
3.0	0.0

Heat resistance of the organisms present on a portion of the ham sample as determined by the end-point technique is listed in Table 2.

Spoilage visible in the processed tubes held at 32–36° C corresponds to the results shown in Table 2. Low numbers of spores remain viable in the product after the nonsterile process. Germination and growth of these spores will occur after many months if they are removed from the meat and placed in broth. If they are allowed to remain in the ground ham they eventually are unable to divide and grow. The *Bacilli* spores are killed between a process of F_o 0.2 and F_o 0.6. Spores surviving the F_o 0.6 and F_o 1.0 process were largely *Clostridia* of the PA3679 type.

To determine predominant types of organisms 100 hams were sampled using the same sampling procedure over a 10-day period. Counts were made on different types of media from the composite sample. Numbers and types of organisms are shown in Table 3. Ingram and Hobbs *(8)* found similar numbers of oragnisms and made complete identification of the cultures.

TABLE 3

Numbers and types of ham natural contaminants when plated on different media

Media	Count bacteria/gram	Type of organisms
North's gelatin agar	20,000	Predominantly cocci with several types of rods
Dextrose, tryptone, yeast extract agar	26,000	Cocci and bacilli
Malt extract agar	2,000	Cocci
Brilliant green agar	.....	.....
A.P.T. (Niven's media)	9,000	Cocci
Potato dextrose agar	10	Short rods
Stone gelatin agar	20,000	Cocci
Phenol red mannitol agar (7.5% NaCl)	16,000	Cocci
Nutrient gelatin	10,000	Cocci and bacilli
Pasteurized count (150° F) (Eugon agar)	12	Clostridia Cocci Bacilli

Tubes were made from the composite ham sample and processed to the F_o values indicated at $z = 18$. After processing, 4 tubes at each level were subcultured with these results:

	Results of Subculture			
F_o	0.05	0.2	0.6	1.0
	positive	positive	positive	positive

The heat resistance coccus from the Eugon agar tubes was isolated and the thermal resistance determined at $z = 9$. The culture when grown in broth survived F^1 50 but was destroyed at F^1 60.

Again it is emphasized that counts made on the hams thus far represent the surface areas and tend to be higher than those for the entire ham. An entire 14-lb ham had a total count of 4,700 organisms per gram and a spore load of 0.4 spore per gram. This spore load agrees with the work of Harriman *et al (6)*.

Most hams processed as non-sterile and labeled "Perishable, Keep Under Refrigeration" contain both spores and viable cocci. The Meat Inspection Division of the Department of Agriculture requires, in the processing of this product, a minimum internal temperature of 150° F. We calculated the process now used at the Ottumwa plant on 3 different sized canned hams (4 lb, 5 lb, 6 lb). Calculations were performed using Ball's *(1)* mathematical procedure and the general method of Bigelow, *et al (3)*. Results expressed as F^1 values show the following equivalent minutes at 150° F.

Method	Size of can		
	4 lb	5 lb	6 lb
General method (F^1)	47.6 min	60.0 min	59.6 min
Ball's method (F^1)	32.4 min	39.5 min	40.8 min

These results, which are in general agreement, show that we are processing in excess of MIO requirements. Initial experiments indicated that several types of cocci were present in the canned hams after processing. Only one type vegetative cell from the hams had a resistance above the actual process values when the cultures were grown in broth. These data are given in Table 4 in addition to the heat resistance values from a group of similar cultures sent to us by Dr. J. B. Evans of the American Meat Institute Foundation. Data in Table 4 include cultures from the intestine of the pig since Willingale and Briggs *(13)* found largely *Lactobacilli* and *Streptococci* in the fæces of normal pigs.

TABLE 4

Heat resistance of organisms isolated from canned hams and grown in peptone beef extract broth (Difco) for 48 hours

Strain	Species	Source	Initial count organisms per gram	Minutes at 150° F	
				Survived	Killed
HS-5 [1]	*S faecium*	Holland Ham	8.3×10^8	45	55
HD-4B [1]	*S faecium*	American Ham	3.3×10^7	15	25
HS-6 [1]	*S faecium*	German Ham	8.4×10^8	10	20
ARA-10 [1]	*S faecium*	Pig Gut	1.2×10^8	5	15
C6-1 [1]	*S durans*	Am Ham Co C	8.5×10^7	5	15
A6-1 [1]	*S faecalis*	Am Ham Co A	2.5×10^8	5	10
4931	.....	Morrell Ham	5.1×10^8	45	55
4936	.....	Morrell Ham	2×10^8	1	10
4943	.....	Pig Gut	2×10^8	1	5
5006	.....	Pig Feces	11×10^8	less than 10	10

[1] Cultures supplied by Dr. J. B. Evans, American Meat Institute Foundation.

With a level of cocci in the range of 10,000 to 20,000 or less it should be impossible to isolate viable cocci from canned hams processed to F^1 values of 40–60 if it is assumed that the heat resistance of cells grown in broth is the same as the heat resistance of cells grown in meat. Our initial experiments to determine the heat resistance of cells grown in meat indicated that some of the cocci were able to resist 400 min at 150° F. Data were extremely variable in recovery counts from meats until irradiated (5 megarad) meat digested overnight with 1% bromelin was used as the growth media. Reproducible results were also possible when the heat resistant cocci were allowed to grow in baby food meat. Table 5 presents data on surviving cells after processing when grown in broth and comparable results on the same culture grown in meat. The broth culture appears to have a logarithmic order of death. In meats this is not the case, but presumably this may be due to our inability to separate the cells from the meat substrate. White *(12)* found survivor curves of *Strep. faecalis* and *Strep. lactis* were not always straight lines in her heat resistance work.

TABLE 5

Rate of destruction characteristics of cocci grown in peptone beef extract broth, enzyme digested meat and baby food meat

Broth Initial count 1×10^8		Meat (1% Bromelin) Initial count 9×10^8		Baby food meat Initial count 9×10^8	
F^1	Surviving cells	F^1	Surviving cells	F^1	Surviving cells
1	100,000,000	50	800,000	50	4,000,000
10	11,000,000	100	60,000	100	25,000
20	200,000	200	400	150	335
30	12,000	300	30	200	15
40	2,000	400	155	250	50
50	10	500	0	300	15
60	0	600	0	400	15
70	0	700	0	500	2
				600	1
				700	0

It was not possible to increase the heat resistance of the culture by selection using isolated colonies from cells able to survive F^1 400. To determine whether the increase in resistance was due to a protective effect of meat, the heat resistant cocci were grown in broth and the cells centrifuged, washed and transferred to 0.0625 M phosphate buffer and meat. Heat resistance values were similar indicating that the growth media and not suspending media contributed to increased resistance.

The heat resistant coccus was inoculated into sterile irradiated pork to determine its ability to grow in meat at low temperatures. The following data were obtained:

	40–50° F	34–38° F
Initial count	2.4×10^5	2.4×10^5
1 day	5.1×10^5	4.1×10^5
2 days	4×10^6	4.3×10^6
3 days	4×10^6	7.0×10^6
5 days	1.4×10^7	1.4×10^6
7 days	2×10^7	3×10^6
14 days	2×10^7	2×10^6

The heat resistant cocci present in the ham after processing were able to grow slowly at refrigeration temperatures. Jepsen (1941) also found many micrococcus strains from meat able to grow at 2° C. A sharp reduction in spore load has been observed when the cocci are present as a high percentage of the total flora. It appears that the cocci present in meat act as a safety measure against the growth of organisms that may be undesirable.

DISCUSSION

The outgrowth of spores present in meat at an extremely low level is very interesting. Unpublished data from this laboratory indicate that the spores present as natural contaminants are probably a small number of ungerminated spores from a larger population of spores that have germinated. Meat contains all of the essential germination requirements for spores. Under optimum conditions in the laboratory 100% germination is extremely difficult or impossible. A small number of spores remain in the dormant or ungerminated state. Data from this laboratory indicate that curing salts and/or other types of inhibitors will prevent or retard these spores from germinating and growing. On the other hand, if we add an equal number of spores that have been grown in laboratory media to cured ham, immediate germination and growth takes place. Additional work is needed to elucidate the differences observed between spores found as natural contaminants and spores added to the meat from culture collections.

SUMMARY

The heat resistance of spores and vegetative cells found on hams prior to canning has been studied. Most *Bacilli* spores are destroyed before a process of F_0 0.6 is reached. Small numbers of anaerobic spores are able to withstand a process of F_0 1.0 but are destroyed before the heating level has reached F_0 3.0. A cocci culture isolated from semi-perishable ham is able to withstand a process of 40 minutes at 150° F. The same culture when grown in meat can survive a process of 400 minutes at 150° F. The relationship between the types of organisms present after processing in semi-perishable hams is discussed.

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VISCOMETRY OF NON-NEWTONIAN FOOD MATERIALS[a]

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The consistency of food materials has frequently been used to measure quality and to predict the changes that might occur under certain conditions. At the present time, most of the devices used to measure the consistency of food materials show results that are expressed in terms of viscosity. However, it has been observed that viscosity varies with rate of flow, in such fluid foods as purees or emulsions, as well as with other conditions.

Those fluids which maintain a constant consistency irrespective of velocity are known as Newtonian. It is impossible to avoid the confusing aspects of the variable consistency of non-Newtonian fluids (in which consistency changes with velocity) without considering shear stress and rate of shear relationships.

For a fluid between two parallel plates, with an area A (Fig. 1) and a force F applied to one of the plates, F/A is considered the shear stress. The plate moves with a velocity V and the distance between the plates, "R", is sufficiently small that the velocity gradient is a constant, and "$-V/R$" $= -dV/dR$ which is the rate of shear.

If the plot of F/A vs. $-V/R$ or $\left(\frac{-dV}{dR}\right)$ is a straight line passing through the origin, then the fluid is Newtonian. The slope of the line is the viscosity coefficient or viscosity, (Fig. 1). If the plot does not exhibit a straight line passing through the origin, the fluid is non-Newtonian.

The equation expressing the shear stress-rate of shear relationship of Newtonian fluids is given by equation (2-1).

$$\tau = \mu\left(-\frac{dV}{dR}\right) \qquad (2\text{-}1)$$

where: τ = Shear stress

$-\left(\frac{dV}{dR}\right)$ = rate of shear

μ = viscosity coefficient or viscosity

The use of the power equation has been suggested by several authors *(4)*, for relating the shear stress and rate of shear of non-Newtonian materials. These are applied to fluids that are not time-dependent as follows:

a.) Bingham plastic

$$\tau = b\left(-\frac{dV}{dR}\right) + C \qquad (2\text{-}2)$$

where: b = Proportionality factor
C = yield stress

Contribution No. 343 from the Department of Food Technology.

b.) Pseudoplastic

$$\tau = b\left(-\frac{dV}{dR}\right)^{s}; \quad 0 < s < 1 \tag{2-3}$$

where: s = pseudoplasticity constant

c.) Dilatant

$$\tau = b\left(-\frac{dV}{dR}\right)^{s}; \quad 1 < s < 0 \tag{2-4}$$

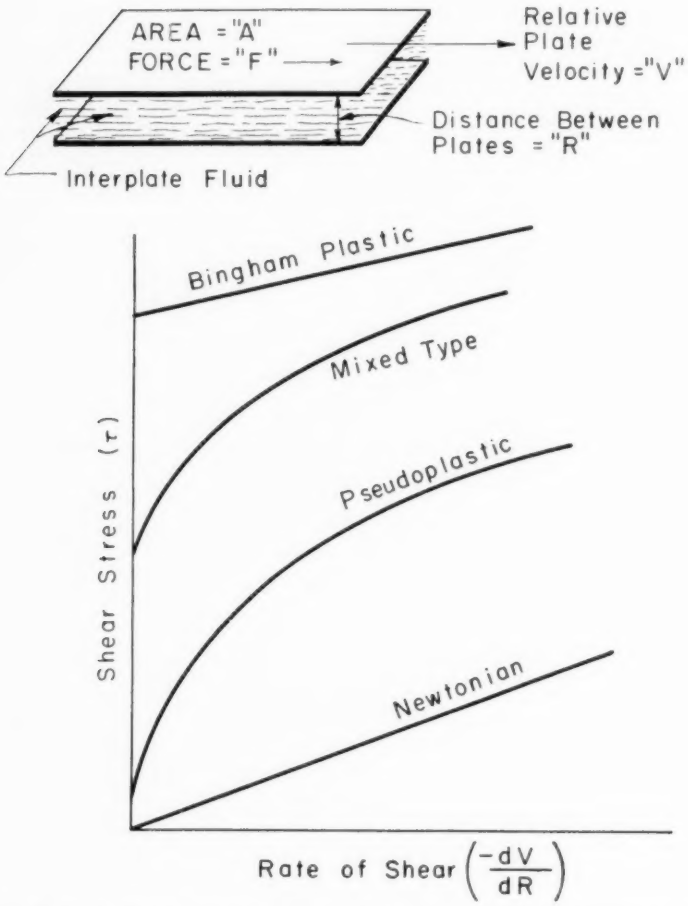

Figure 1. Theoretical system for shear stress—rate of shear measurements and shear stress—rate of shear relationships for various types of fluids.

It is possible to consider the general equation for all classic non time-dependent materials as follows:

$$\tau = b\left(-\frac{dV}{dR}\right)^s + C \qquad (2\text{-}5)$$

The shear stress-rate of shear relationships of many non time-dependent food materials may be expressed by equation (2-5).

Some fluids are considered to be time dependent, in the sense that the constants, b, s and c vary with time *(4)*.

Thixotropic fluids may be thought of as pseudoplastics at any time. Rheopectic fluids may be considered as dilatant at any time. The power equations as expressed have no theoretical basis. They are simply general, empirical equations which approximate the shear relationships over a wide range. By classifying food materials according to empirical shear stress-rate of shear relationships, it is possible to calculate frictional loss in tubes, e.g. *(3)*, and heat transfer coefficients *(1)*, and mixing and coating characteristics for non-Newtonian fluids.

The constants b, s, and C may be evaluated by means of a tube type viscometer or by a specially designed narrow-gapped coaxial viscometer *(2)*. Contrary to reports in the literature, it is not possible to employ most of the conventional rotational viscometers for this purpose, except in the case of Newtonian materials.

Narrow-gapped coaxial viscometers permit a more direct measurement of the constants b, s and C than do tube viscometers, but they are limited by particle size and are expensive.

By employing a poiseuille type analysis to the flow in a tube, it is possible to derive an expression for materials which behave according to Equation (2-5). This expression relates the pressure drop and length and rate of flow to constants b, s, and C.

In a circular tube of radius R_w and length ΔL consider a cylinder of fluid with radius R, measured from the center, and a length ΔL.

The force impelling the element of fluid to move downstream is $\Delta P \pi R^2$ where ΔP is the pressure difference in the tube. The force retarding this movement is $\tau(2\pi R \Delta L)$ or the shear force acting on the curved surface of the cylinder.

Since the fluid is flowing with a constant velocity, the net forces acting on the cylinder of fluid must be zero and

$$\Delta P \pi R^2 = 2\pi R \Delta L(\tau)$$

$$\text{since } \tau = b\left(-\frac{dV}{dR}\right)^s + C; \quad \text{(see Equation 2-5)}$$

$$\Delta P \pi R^2 = \left[b\left(-\frac{dV}{dR}\right)^s + C\right](2\pi R \Delta L) \qquad (2\text{-}6a)$$

If it is assumed that there exists at the tube wall a very thin film with zero velocity, it is possible to rearrange Equation (2-6a) as follows:

$$-\frac{dV}{dR} = \frac{1}{b^{1/s}} \qquad \left[\frac{\Delta P R}{2\Delta L} - C\right]^{(1/s)} \qquad (2\text{-}6b)$$

and

$$\frac{1}{b^{1/s}}\int_{R}^{R_w}\left[\frac{\Delta PR}{2\Delta L}-C\right]^{1/s}dR=\int_{V}^{0}dV \tag{2-6c}$$

or

$$V=\frac{2\Delta L}{\Delta P b^{1/s}}\left[\frac{\left(\frac{\Delta PRw}{2\Delta L}-C\right)^{1/s+1}-\left(\frac{\Delta PR}{2\Delta L}-C\right)^{1/s+1}}{1/s+1}\right] \tag{2-7a}$$

Equation (2-7a) represents the point velocity profile existing in the tube when the layers of fluid are slipping past one another in an orderly manner. It is also assumed in Equation (2-7a) that the yield stress, C, has been exceeded at all points, otherwise negative velocity would appear to occur at some points in the tube. It may be observed from Equation (2-6b) then that when $-dV/dR$ is equal to zero, $R=\frac{2\Delta LC}{\Delta P}$ At this value of R the velocity will be maximum. At $R=\frac{2\Delta LC}{\Delta P}$, Equation (2-7a) becomes discontinuous and at the point between $R=\frac{2\Delta LC}{\Delta P}$ and $R=0$ the velocity remains constant at the maximum value (see Fig. 2). When $C=0$, the maximum velocity occurs at the tube center. (Figure 2)

For points between $R=R_w$ and $R=\frac{2\Delta LC}{\Delta P}$ Equation (2-7a) may be applied. For all points between $R=\frac{2\Delta LC}{\Delta P}$ and $R=0$, velocity is constant and is the maximum velocity in the tube. The maximum velocity may be determined by means of the following equation:

$$V_{max}=\frac{2\Delta L}{\Delta P b^{1/s}}\left(\left[\frac{\Delta P}{2\Delta L}Rw-C\right]^{1/s+1}\right)\frac{1}{1/s+1} \tag{2-7b}$$

The total volumetric rate of flow in a tube may be determined by considering the flow rate in two parts: first, in that region where the yield stress is exceeded and, second, where the yield stress has not been exceeded.

For the region where the yield stress is exceeded, the volumetric rate of flow may be expressed by

$$Q_1=\int_{\frac{2\Delta LC}{\Delta P}}^{R_w}V2\pi RdR \tag{2-8a}$$

or substituting Equation (2-7a) for V and integrating

$$Q_1=\frac{2\pi}{(1/s+1)\left(\frac{\Delta P}{2\Delta L}\right)b^{1/s}}\left[\frac{\left(\frac{\Delta P}{2\Delta L}R_w-C\right)^{1/s+1}}{2}\left[R_w^2-\left(\frac{2\Delta LC}{\Delta P}\right)^2\right]-\frac{\left[R_w\left(\frac{\Delta P}{2\Delta L}\right)(1/s+1)+C\right]\left[\frac{\Delta P}{\Delta L}R_w-C\right]^{1/s+2}}{\left(\frac{\Delta P}{2\Delta L}\right)^2(1/s+3)(1/s+2)}\right] \tag{2-8b}$$

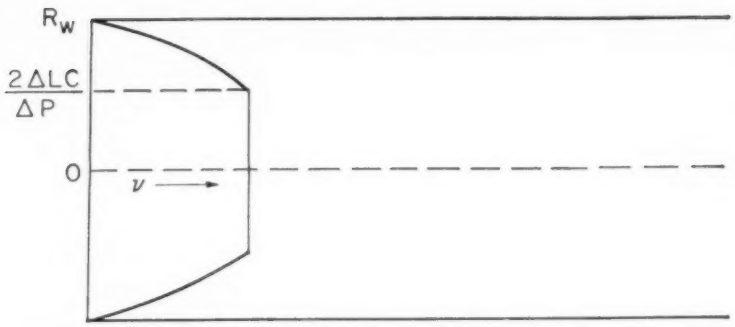

Figure 2. Velocity distribution in tube of fluid with yield point.

The volumetric rate of flow of the remaining central core of fluid, all of which is moving at the maximum velocity, is

$$Q_2 = V_{max} \int_0^{\frac{2\Delta LC}{\Delta P}} 2\pi R dR \tag{2-8c}$$

or substituting Equation (2-7b) for V_{max} and integrating,

$$Q_2 = \pi \left(\frac{2\Delta LC}{\Delta P}\right)^2 \left[\frac{2\Delta L}{\Delta P b^{1/s}}\right] \left[\frac{\Delta P R_w}{2\Delta L} - C\right]^{1/s+1} \frac{1}{1/s+1} \tag{2-8d}$$

The total volumetric rate of flow is

$$Q \text{ total} = Q_1 + Q_2 \tag{2-8e}$$

When $C = 0$

$$Q \text{ total} = \left(\frac{\Delta P}{2b\Delta L}\right)^{1/s} \left(\frac{\pi}{1/s+3}\right) (R_w^{1/s+3}) = \bar{V}\pi R_w^2 \tag{2-8f}$$

Where $\bar{V}$ = mass average Velocity

2-2 Experimental evaluation of flow constants b, s and C. By applying experimental volumetric rate of flow and pressure drop information obtained from a tube type viscometer, it is possible to calculate the flow constants b, s and C (Figure 3.)

For pseudoplastic, Newtonian and dilatant fluids in which $C = 0$, the constant "s" may be determined by plotting log Q vs. log $\Delta P/\Delta L$. The slope of the resulting line is 1/s. The constant "b" may then be calculated after determining "s" by solving Equation (2-8f) (see Example 2-2).

To determine the flow constants for fluids in which "C" is not zero, it is first necessary to establish the value of "C". In order to do this, it is first necessary to plot experimental values of Q vs. $\Delta P/\Delta L$ and to extrapolate this to $Q = 0$. If $(\Delta P/\Delta L)$ does not equal zero at this point also, there must be a yield stress in the fluid (see Fig. 4).

If $(\Delta P/\Delta L)_0$ is the maximum or extrapolated value of $\Delta P/\Delta L$ when $Q = 0$, then according to Equation (2-6b),

$$\left(\frac{\Delta P}{\Delta L}\right)_0 \frac{1}{2C} = \frac{1}{R_w} \tag{2-9a}$$

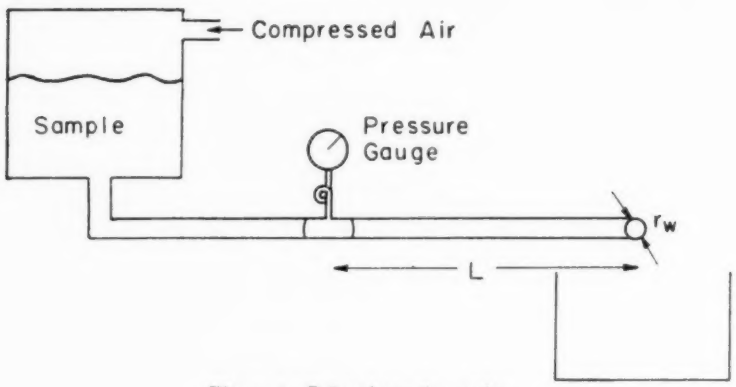

Figure 3. Tube viscometer system.

From Equation (2-9a), it is possible to solve for C (see Example 2-1). The flow constants "b" and "s" may be determined by solving Equation (2-8c) simultaneously with two different experimental values of Q total and $\Delta P/\Delta L$. This is done most conveniently by assuming a value of s and evaluating $b^{1/s}$. A plot may be made of $b^{1/s}$ vs. the assumed values of s and a set of curves for each value of Q total and $\Delta P/\Delta L$ may be obtained. The point intersection of these curves determines the correct value of "s" and "b" (see Figure 5 and Example 2-1).

Example (2-1)

The testing of fluid in a tube viscometer gave the pressure drop volumetric rate of flow information listed in Table I. The tube of the measuring section of the viscometer is 122 cm long and 1.27 cm inside diameter. Determine the flow constants b, s and c for the fluid.

TABLE 1

$\frac{Q}{(cm^3/sec)}$	$\frac{\Delta P}{(dynes/cm^2)}$	$\frac{\Delta P/\Delta L}{(dynes/cm^2)}$
0.1	183,000	1,500
0.5	275,000	2,250
1.3	348,000	2,850
4.3	483,000	3,900
9.9	622,000	5,100

Solution

The data in Table 1 are plotted in Figure 4. The curve in Figure 4 is extrapolated to $Q = 0$, and $(\Delta P/\Delta L)_0$ corresponding to this 1000 dynes/cm^3 From Equation (2-9a)

$$2\left(\frac{\Delta L}{\Delta P}\right)_0 C = R_w = 0.635 \text{ cm}$$

$$C = 317.5 \text{ dynes/cm}^2$$

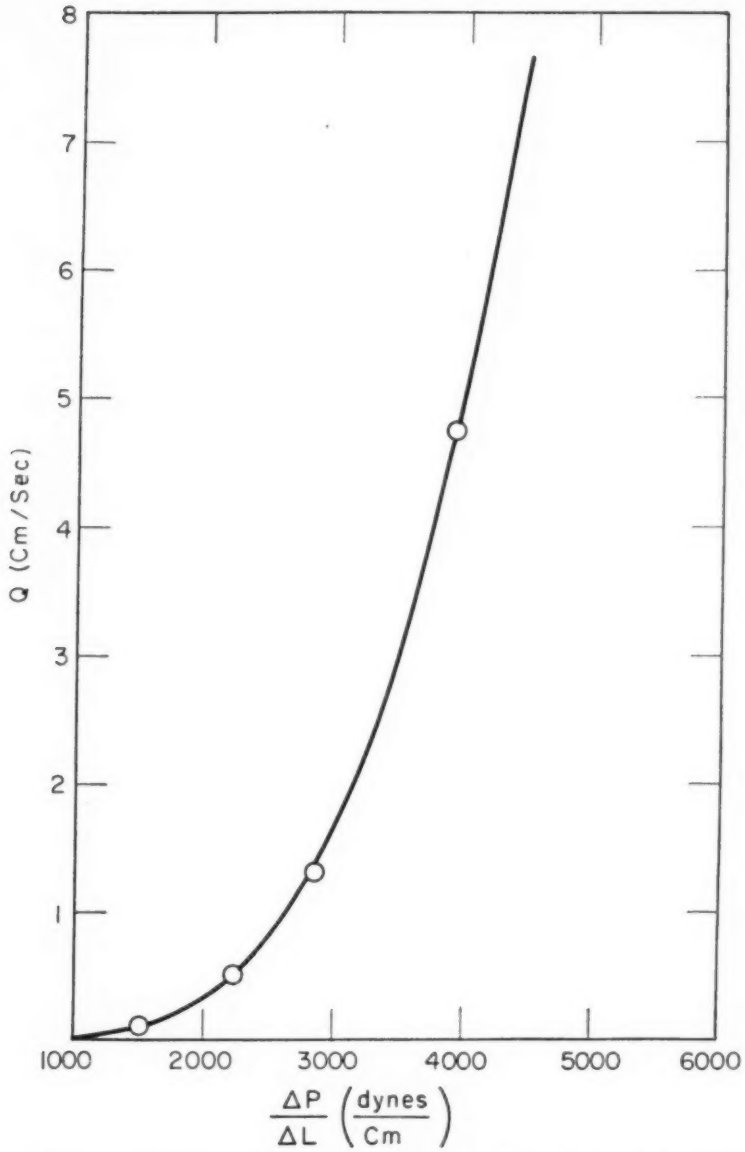

Figure 4. Volumetric rate of flow vs pressure drop per unit length for fluid with yield stress.

TABLE 2

$\frac{Q}{(cm^3/sec)}$	$\frac{\Delta P/\Delta L}{(dynes/cm^3)}$	s	$\frac{b^{1/s}}{(dynes/cm^2)^{1/s}(sec)}$
.1	1500	.5	7.8×10^6
		1.0	2.69×10^2
		2.0	2.26×10^1
		0.4	5.53×10^3
9.9	5100	.5	6.86×10^6
		1.0	7.18×10^2
		2.0	2.53×10^{-1}

Considering the condition where $Q = 0.1$ cm³/sec and $\Delta P/\Delta L = 1500$ dynes/cm³, "$b^{1/s}$" is calculated by trial and error for various assumed values of "s" using Equation (2-8c). In Table 2 the results are tabulated.

Then considering the case where $Q = 9.9$ cm³/sec and $\Delta P/\Delta L = 5100$ dynes/cm³, again $b^{1/s}$ is calculated for various assumed values of "s" (see Table 2).

From the results in Table 2 it is possible to plot $b^{1/s}$ vs assumed value of "s" for the two conditions of $\frac{\Delta P}{\Delta L}$ (Fig. 5). The intersection of the two curves located the correct value of "s" and $b^{1/s}$. From Figure 5 it appears that

$$s = 0.450$$

and

$$b = 2.29 \times 10^2 \frac{\text{dynes (sec)}^{.450}}{\text{cm}^2}$$

Thus the flow constants for the fluid are:

$$s = .450$$

$$b = 2.29 \times 10^2 \frac{\text{dynes (sec)}^{.450}}{\text{cm}^2}$$

$$C = 317.5 \text{ dynes/cm}^2$$

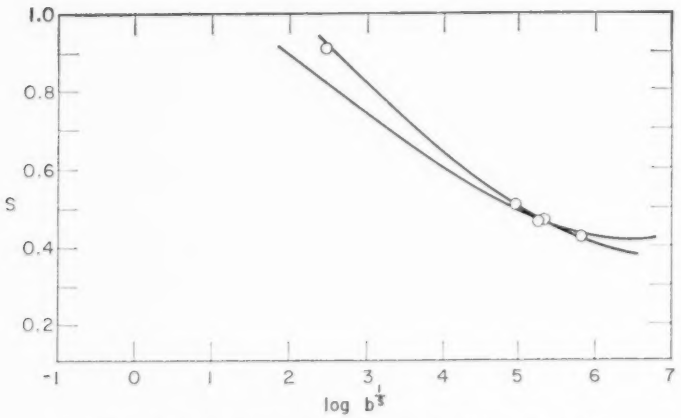

Figure 5. s vs log b 1/s for two different conditions of $\Delta P/\Delta L$.

TABLE 3

Q	$\frac{\Delta P}{\Delta L}$
$\frac{(cm^3)}{(sec)}$	$\frac{(dynes)}{cm^3}$
1.13×10^{-2}	1.265×10^3
2.00×10^{-2}	2.300×10^3
3.10×10^{-2}	3.450×10^3
4.17×10^{-2}	4.95×10^3
6.25×10^{-2}	7.24×10^3

Example (2-2)

Determine the flow constants b, s and C for blood from the rate of flow and pressure drop information given in Table 3. The diameter of the tube is .03 cm. and the length is 2 ft.

Solution

From Figure 6, it is noted that $(\Delta P/\Delta L)_0 = 0$ and therefore $C = 0$.

Since $C = 0$, it is possible to determine "s" by plotting log Q vs. log $\Delta P/\Delta L$ (see Fig. 7). The slope of the line is $\frac{1}{s}$. From Figure 7 it is found that "s" is 0.890.

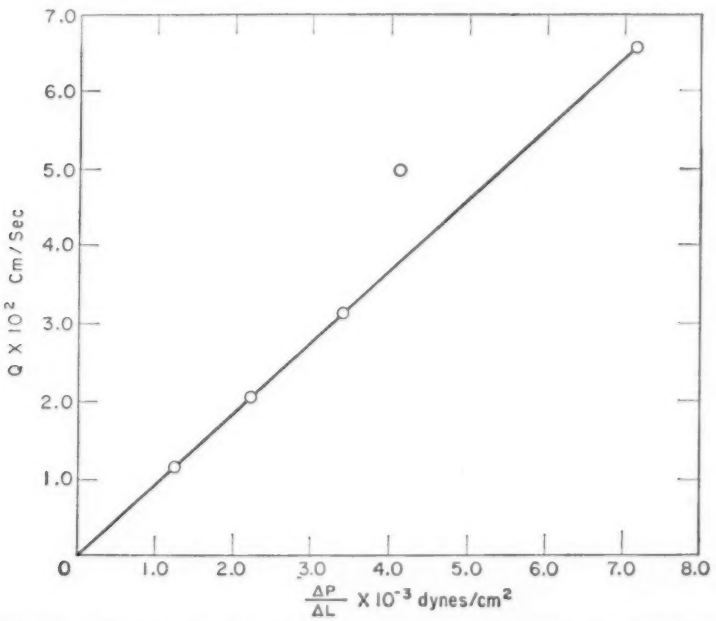

Figure 6. Volumetric rate of flow vs pressure drop per unit length for human blood.

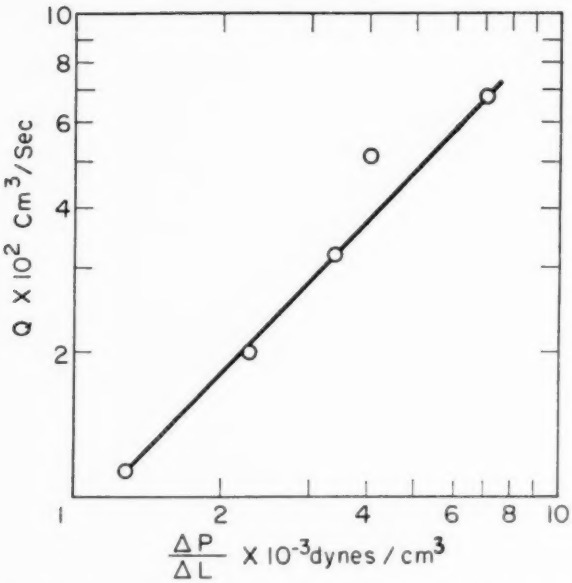

Figure 7. Log volumetric rate of flow vs log pressure drop/ unit length for human blood.

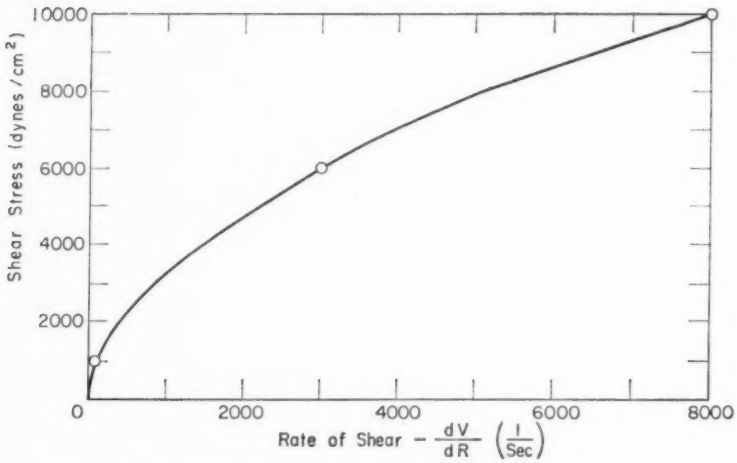

Figure 8. Shear stress vs rate of shear 3.37% ammonium alginate 75° F.

To solve for "b", Equation (2-8f) is employed for the condition where $Q = 3.10 \times 10^{-2}$ cm³/sec and $\Delta P/\Delta L = 3.45 \times 10^{3}$ dynes/cm³.

$$3.10 \times 10^{-2} = \left(\frac{3.45 \times 10^{3}}{2b}\right)^{1/0.890} (1.5 \times 10^{-2})\,(1/.89) + 3$$

$$b = 3.84 \times 10^{-3} \left(\frac{\text{dynes}}{\text{cm}^2}\right) (\text{sec})^{.89}$$

Therefore the flow constants for human blood at 80° C are

$$C = 0$$

$$b = 3.84 \times 10^{-3} \left(\frac{\text{dynes}}{\text{cm}^2}\right) (\text{sec})^{.89}$$

$$s = 0.890$$

TABLE 4
Flow constants for various types of materials

Material	Temp. (° F)	Instrument	b (dynes/cm²) (sec)ˢ	s	C (dynes/cm²)
Apple Sauce Brand A	75° F	Merrill[1] Viscometer	5.00	0.645	0
Apple Sauce Brand B	75° F	Tube[3] Viscometer	6.60	0.408	0
Banana Puree Brand A	75° F	Merrill Viscometer	65.0	0.458	0
Banana Puree Brand B	75° F	Tube Viscometer	107.	0.333	0
Honey	75° F	Brookfield Viscometer	56.0[2]	1.00	0
Honey	75° F	Tube Viscometer	61.8[3]	1.00	0
3.3% Ammonium Alginate	75° F	Merrill Viscometer	135.	0.477	0
3.3% Ammonium Alginate	75° F	Tube Viscometer	125.	0.500	0
Human Blood	80° F	Tube Viscometer	3.84×10^{-3}	0.890	0

[1] Merrill coaxial narrow-gapped viscometer, radius of outer cylinder 2.601 cm and a gap between cylinders of .01 cm. The outer cylinder remained stationary while the inner cylinder rotated. See reference 2.

[2] Since c = 100, honey is Newtonian and the measurement is in terms of poises.

[3] Tube Viscometer employed compressed air to force fluid through tube. Pressure gauge measured P. See (1).

2-4 Consistency

The ratio of $\tau / \frac{-dv}{dR}$ may be considered to be the consistency of the fluid. It appears from an examination of Figure 8 that the consistency will vary with velocity. For example, a 3.37% ammonium alginate solution at very low rates of shear, e.g. 100 1/sec, has a $\tau / \frac{-dv}{dR}$ of 1000/100 or 10 poises if "τ" is expressed in dynes/cm^2. At 8000 1/sec, $\tau / \frac{-dv}{dR}$ is 10,000/8000 of 1.25 poises. The flow constants are not functions of the rate of flow but are properties of the fluid.

In Table 4 the flow constants of various food fluids are listed.

SUMMARY

A method is described for establishing the flow constants of non time-dependent, non-Newtonian food materials. The procedure employs an empirical generalized relationship between shear stress and rate of shear. The equation is adaptable to materials which have a yield point as well as those which have not.

The determination of the fluid flow constants as outlined, permits the calculation of heat transfer and flow characteristics for various conditions.

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METHODS OF EVALUATING FREEZE-DRIED BEEF[a,b]

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There has been a great improvement recently in the quality of freeze-dried beef, as determined by taste panels, but very few attempts have been made to assess the type or extent of the changes which may result from the freeze-drying process. Such information would, *inter alia*, provide an objective method of evaluating drying techniques and aid in the preparation of better freeze-dried products. In addition, objective physico-chemical tests would probably be more sensitive and more reliable than taste panel appraisals.

The adenosine triphosphatase (ATPase) activity and contractility of the fibrils from freeze-dried beef, prepared by the modified Vacuum Contact Drying (V.C.D.) process *(11)*, has been reported by Hunt and Matheson *(6)*. They found that 20 to 60% of the activity and some of the contractility had been lost during drying. We were interested first in confirming those findings, and secondly in making a more intensive survey of the effect of various freeze-drying methods. Hence, in this study, an attempt has been made to determine (a) what criteria might be applied as a means of assessing effectively the characteristics of freeze-dried beef, (b) whether or not there are great differences between freeze-dried and frozen beef when these criteria are applied, (c) whether or not differences in the drying techniques of freeze-dried beef are reflected in the results of physico-chemical tests.

Because contractility is difficult to quantify the criteria investigated have been salt solubility, ATPase activity, and electrophoretic mobility. Some cursory observations are also reported on the sedimentation behaviour of the fraction extracted by strong salt solution.

Muscle proteins may be divided into four groups, according to their solubility:

1. Corpuscular proteins, soluble in dilute salt solution (of the order of 0.15M) at neutral pH. These are largely enzymic in nature and include the haemproteins.

2. Proteins of the muscle fibre—so far established as a complex of myosin, actin and tropomyosin, and soluble in stronger salt solution (ionic strength about 0.6).

3. Proteins soluble in 0.1N NaOH, and insoluble in high concentrations of salt solution.

4. Collagen and elastin (connective tissue), insoluble in 0.1N NaOH.

With pre-rigor muscle, or muscle obtained from animals which have been freshly killed, the proteins of group 2 are obtained by extracting directly with stronger salt solution and precipitating by dilution to an ionic strength of about 0.05. Since it was desired to determine the effect of freeze-drying on those

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[a] Defence Research Medical Laboratories Report No. 232-4 PCC Project No. D50-78-03-01.

[b] Presented at the Nineteenth Annual Meeting of the Institute of Food Technologists, May 19, 1959, Philadelphia, Pennsylvania.

protein fractions of post-rigor muscle which are most likely to undergo change, namely, group 1 and 2, the latter procedure would prove cumbersome as a routine analytical procedure. In this study therefore fractionation was carried out in a stepwise manner from lower to higher ionic strength solution, on the same sample of beef.

EXPERIMENTAL

Raw material. The *semitendinosus* muscle from 3 animals was used in this study; they are referred to hereinafter as *semitendinosus* I, II, and III. The dressed weight and hanging time of the carcasses of *semitendenosus* II and III were, respectively, 432 lb, 26 days and 672 lb, 14 days. These data were not available for *semitendinosus* I.

After excision the entire *semitendinosus* I muscle was minced to pass a ⅛ in. plate. Only a portion of the other 2 muscles was minced. The minced portion and the remaining muscle were frozen in air at −15° C. The frozen muscle was cut into ½ in thick slices and returned to the freezer as quickly as possible. The weight of each sample was taken before and after drying.

Drying techniques. Conventionally, freeze-drying is carried out in a chamber maintained at about 1 mm Hg pressure, in which the meat is laid on a shelf maintained at about 40° C. This process requires up to 24 hr for completion. The drying time has been reduced, however, by recent techniques which employ devices for improving heat transfer *(1, 2, 11)*.

In this study 4 different techniques were used for drying the frozen muscle.

1) The muscle, minced or in slices, was laid on a heated shelf for the required length of time, with the chamber pressure maintained between 400 and 600 mc Hg. *Semitendinosus* I was dried at a shelf temperature of 30° C for 30 hr; *semitendinosus* II and III were dried at a shelf temperature of 37° C and 30° C, respectively, for a period of 20 hr each.

2) Slices were dried using spiked plates *(2)* at 500 mc Hg pressure. Maximum plate temperature in the final stages was 50° C and the total time was 5 hr.

3) Slices were prepared by the V.C.D. technique *(11)* and required 5 hr to dry. Shelf temperature for the first 2 hr was between 90° and 100° C; for the final 3 hr it was maintained at 60° C.

4) Slices were dried with radiant heat from heated blackened shelves *(13)* for 6 hr, a final temperature of 50° C being attained. Chamber pressure in techniques 3 and 4 was 500 mc Hg.

Extracting solutions. Solution 1: ionic strength mc = 0.15; pH 7.4; 0.048M $Na_2HPO_4 \cdot 2H_2O$, 0.006M KH_2PO_4.

Solution 2: *(5)* mc = 0.53; pH 8.25; 0.5M KCl, 0.03M $NaHCO_3$.

All solutions were made up with double distilled water, with the final distillation from glass vessels.

Adenosine triphosphatase (ATPase) activity. The final concentrations of reactants were as follows:

ATP 0.004M; (disodium salt, Sigma)
Calcium chloride 0.02M
Potassium chloride 0.10M
Tris (hydroxymethyl) aminomethane 0.066M
Muscle extract 0.10 to 0.65 mg N/ml.

Determinations were carried out in 15 ml centrifuge tubes at 30° C, the reaction being stopped after 10 min by adding 5 ml of 10% trichloracetic acid. Tubes were centrifuged and suitable aliquots taken for free phosphorus determinations using the method of King *(9)*. The blue colour was read in a Coleman Junior spectrophotometer at 660 mmc after 5 min. A blank determination was run with each series of tests. Perchloric acid was substituted for sulphuric acid to shorten the time required for colour development. The final volume in each case was 5 ml. Hence the calcium activated ATPase activity was determined at pH 9.1 on the extract obtained by solution 2, and is reported as mcg P. liberated/min/mgN.

Extraction procedure. Specimens of minced beef weighing 7.0 g, and freeze-dried beef of the dry weight equivalent were comminuted in 50 ml solution 1 in a steel blender with ½ min burst and 3 min rest periods and repeated 3 times. The tempera-

ture was kept below 4° C throughout. The suspension was centrifuged at 1600*g* for 15 min, separating a reddish brown supernatant from a well macerated tissue brei. Two further extractions of the brei were made, each with 50 ml of solution, by stirring with a glass rod and centrifuging at 1600*g* for 15 min. The total extract was filtered through Whatman filter paper No. 12 and labelled Ext I. The macerated muscle tissue was washed once with 50 ml water to remove an excess of $PO_4^{=}$ ions, and then centrifuged as before. Invariably swelling resulted.

Three extractions with 50 ml solution 2, each of 10 min duration, were made by thoroughly dispersing the macerated pellet with a glass rod, recentrifuging for 15 min at 1600*g*, and decanting the supernatant. The supernatant usually showed layers of obviously differing viscosity, but the entire supernatant was decanted except for the very viscous layer near the pellet. Supernatants were combined, subjected to a final centrifuging for 15 min at 1600*g*, and labelled Ext II.

Since it had been reported *(6)* that all of material extracted by 0.5M potassium chloride from freeze-dried cod could be sedimented at 16,000*g*, it was thought to be of interest to examine some of the sedimentation characteristics of the beef extracts. Preliminary experiments indicated that much of the actomyosin remained in solution at high "g" forces. Consequently a value of 50,000*g* was arbitrarily chosen as suitable to make a cursory appraisal of frozen and freeze-dried beef extracts.

For purposes of comparison, a sample each of minced frozen, and freeze-dried minced beef (*semitendinosus* III) was extracted directly with solution 2. The ionic strength of the extract was reduced to 0.05 and after 4 hr the resulting actomyosin precipitate was separated by centrifuging at 1600*g* for 30 min. The actomyosin precipitate, redispersed in mc 0.53 buffer, was then analyzed for total nitrogen and ATPase activity. Results are given in Table 1.

TABLE 1

Extractable nitrogen and ATPase activity of whole extract and precipitated actomyosin (Semitendinosus III)

	% N extracted by solution 2	ATPASE activity mcg P/min/mg N	
		Whole extract	Actomyosin
Frozen minced beef	81.0	28.4	49.3
Freeze-dried minced beef	80.6	28.3	49.1

Electrophoresis. Electrophoresis was carried out in a Spinco Model H electrophoresis diffusion apparatus. Each extract was dialysed against its own buffer and electrophoretic determinations were made after centrifuging the dialysates until they were completely clear.

RESULTS AND DISCUSSION

After pre-rigor muscle is extracted by weak salt solutions the residue is considered unsuitable for treatment by stronger salt solution if a quantitative measurement is to be made *(3)*. Since there is only a 3.7% decrease in the amount of extractable nitrogen (cf. Tables 1 and 4) when frozen minced beef was treated in the stepwise fashion it appears that the foregoing does not hold

TABLE 2

Extractable nitrogen and ATPase activity of beef before and after dehydration (Semitendinosus I)

	% total N extracted [1]		ATPase activity [1] mcg P/min/mg N
	Solution 1	Solution 2	
Frozen minced beef	39.6	31.3	48.6
Freeze-dried minced beef	37.4	31.2	48.3

[1] Results are the averages of at least 4 determinations.

entirely true for post-rigor muscle, and it is possible to extract virtually all the actomyosin in this way. Further, such deformities of the protein as may be introduced by freeze-drying appear to be more readily detectable by the stepwise procedure than by removal of sarcoplasmic and fibrillar proteins together, followed by precipitation of the latter. As shown in Table 1, the data for freeze-dried and frozen minced beef extracted with mc = 0.53 solution are identical except for a very small difference in extractable nitrogen. However, when fractionation was carried out by the stepwise procedure differences between frozen (control) and dried samples became apparent (Tables 3 and 4). Differences in extractable actomyosin nitrogen vary

TABLE 3

Extractable nitrogen and ATPase activity of before and after drying (Semitendinosus II)

Drying technique	% total N extracted [1]		ATPase activity [1,2] mcg P/min/mg N	% N recovered [1,3] in supernatant
	Solution 1	Solution 2		
Shelf (minced)	33.2	23.1	50.0	53
Shelf (slices)	33.9	28.9	42.0	
Spiked plate	34.6	32.0	44.0	47
Radiant heating	35.7	32.7	44.0	50
Frozen	35.0	34.9	46.4	63

[1] Averages of at least two determinations with a maximum variation of 1% in extractable nitrogen.
[2] After sedimentation at 1,600*g*.
[3] After sedimentation at 50,000*g*.

between zero (Table 2) and 11.8% (Table 3). The range of variation of extractable sarcoplasmic protein is smaller and shows greater consistency throughout. However, on the basis of salt extraction alone there is not sufficient evidence upon which to state that any one technique is to be preferred, except that prolonged heating at 37° C appears to have a greater effect on the percentage of extractable actomyosin nitrogen.

Electrophoretic patterns of mc 0.15 extracts from the various samples lend support to the foregoing evidence that few changes have been introduced. There is only one obvious difference, except for small quantitative changes, between extracts of the frozen sample and two of the freeze-dried samples (minced shelf dried, and spiked plate), and that is the loss of one peak in patterns of the latter, (Figures 1, 2, 3). The V.C.D. pattern (Figure 4) was almost identical to that of the frozen extract. The pattern for radiant heated material was not taken. Three electrophoretic patterns of mc 0.53

ELECTROPHORETIC PATTERN OF μ0·15 EXTRACT OF FROZEN BEEF.
TIME. 9780 SECS. μ = 0·15
GRADIENT. 8·86 VOLTS/CM. PH. = 7·38

Figure 1. Methods for evaluating freeze-dried beef.

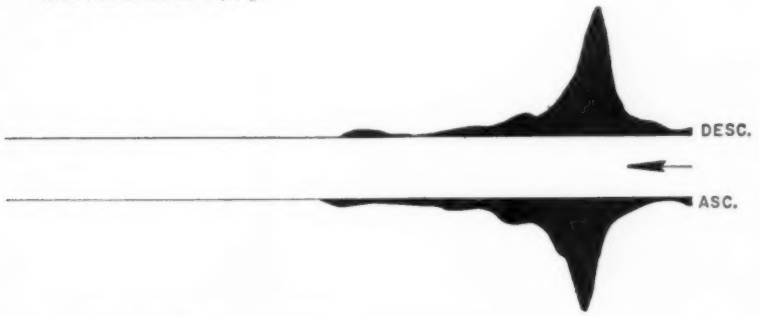
extracts are presented in Figures 5, 6, and 7. The deformity in the descending limb is thought to be caused by the tendency of actomyosin to behave in the electrophoresis tube like a gel *(8)*. As a result of this a certain degree of asymmetry is is introduced and thus the ascending and descending patterns become dissimilar. This effect is particularly evident in the extracts from minced freeze-dried beef; at 35,400 sec (Figure 6) it is quite noticeable, and

TABLE 4

Extractable nitrogen and ATPase activity of beef before and after drying (Semitendinosus III)

Drying technique	% total N extracted [1]		ATPase activity [1,2] mcg P/min/mg N	% N recovered [1,3] in supernatant
	Solution 1	Solution 2		
Shelf (minced)	39.6	30.5	42.3	68
Shelf (slices)	39.4	30.8	39.3	69
Spiked plate	38.8	30.0	36.4	67
V.C.D.	38.8	32.7	35.5	68
Frozen	41.3	35.6	37.5	74

[1] Averages of at least two determinations with a maximum variation of 1% in extractable nitrogen.
[2] After sedimentation at 1,600*g*.
[3] After sedimentation at 50,000*g*.



ELECTROPHORETIC PATTERN OF μ 0·15 EXTRACT OF FREEZE-DRIED MINCED BEEF, PREPARED BY CONVENTIONAL SHELF METHOD.
TIME. 7380 SECS. μ = 0·15
GRADIENT. 8·49 VOLTS/CM. PH. = 7·38

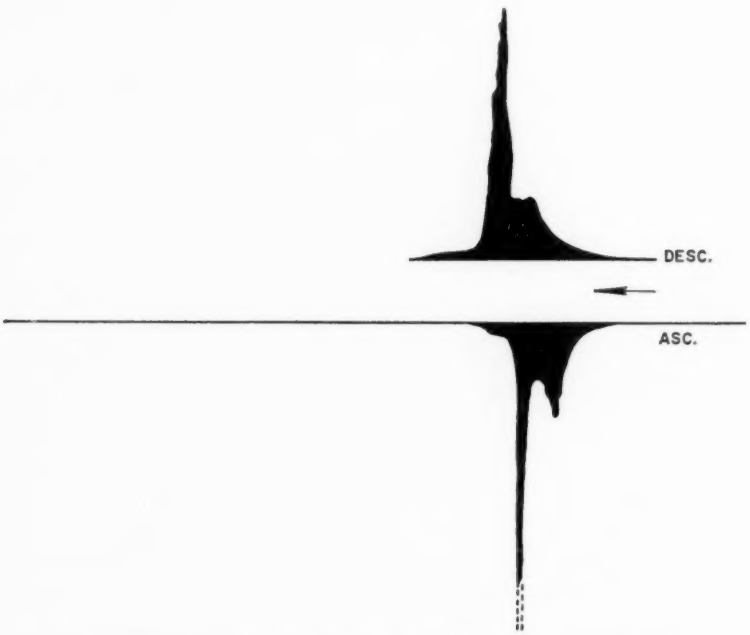
Figure 2. Methods for evaluating freeze-dried beef.

ELECTROPHORETIC PATTERN OF μ 0·15 EXTRACT OF FREEZE-DRIED BEEF PREPARED BY SPIKED PLATE METHOD.
TIME. 9240 SECS. μ = 0·15
GRADIENT. 8·83 VOLTS/CM. PH. = 7·38

Figure 3. Methods for evaluating freeze-dried beef.

ELECTROPHORETIC PATTERN OF μ0·15 EXTRACT OF FREEZE-DRIED BEEF
PREPARED BY V.C.D. METHOD
TIME. 6900 SECS. μ = 0·15
GRADIENT. 8·49 VOLTS/CM. PH. = 7·38

Figure 4. Methods for evaluating freeze-dried beef.



ELECTROPHORETIC PATTERN OF μ0·5 EXTRACT OF FROZEN BEEF.
TIME. 67,850 SECS. μ = 0·5
GRADIENT. 0·78 VOLTS/CM. PH. = 8·1

Figure 5. Methods for evaluating freeze-dried beef.

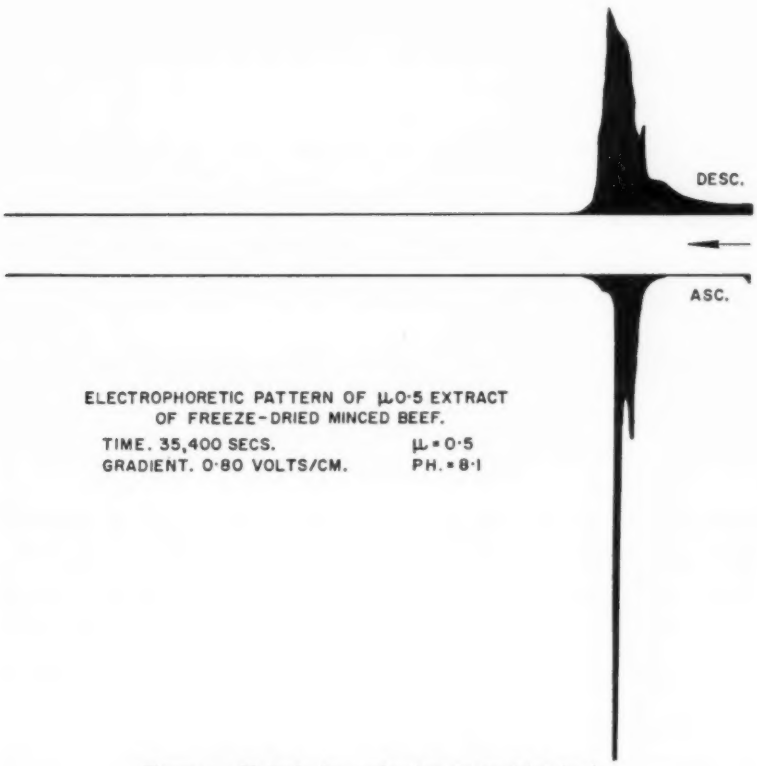


Figure 6. Methods for evaluating freeze-dried beef.

at 74,900 sec the descending pattern is so distorted as to make it unintelligible (only the ascending pattern is shown in Figure 7). Patterns in the ascending limb are, however, very similar in both frozen and freeze-dried material. The greater degree of asymmetry obtained with the freeze-dried sample indicates that some degree of denaturation has occurred, but not sufficient to interfere with the mobility of the proteins.

Electrophoretic mobilities for the peaks shown in Figure 1, of the mc 0.15 extract of frozen beef, expressed in $cm^2/volts/sec \times 10^{-5}$ are 5.95, 3.56, 2.33, 1.35, 0.68, respectively. The mobilities for the components of the mc 0.53 extract cannot be compared with reported values for actomyosin, because electrophoresis was carried out in a higher concentration of buffer and at a higher pH than is usual. The 2 peaks probably represent actomyosin and myosin, with the former in the greater concentration.

It has been reported that all the "soluble" actomyosin of freeze-dried cod may be sedimented at 16,000*g* *(6)*; beef actomyosin on the other hand does not appear to behave in the same way. Actually, a substantial portion may be in true solution, since after subjecting the extract to 105,000*g* for 5 hr,

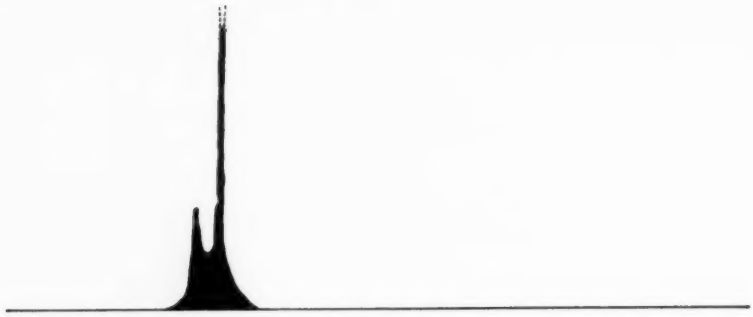

ELECTROPHORETIC PATTERN OF μ 0·5 EXTRACT OF FREEZE-DRIED BEEF
PREPARED BY SPIKED PLATE METHOD
TIME. 74,900 SECS.
GRADIENT. 0·80 VOLTS/CM.
μ = 0·5
PH. = 8·1

Figure 7. Methods for evaluating freeze-dried beef.

20 to 40% of the original nitrogen still remained in the supernatant. Sedimentation results (Tables 3 and 4) show that actomyosin extracts from freeze-dried beef sediment at a faster rate than actomyosin from frozen beef. The reason is as yet uncertain, but the difference may be considered as another index of protein denaturation. These results do not indicate that sedimentation is in any way superior to the other criteria examined but, in view of other recent evidence published by Seagran *(12)* and Connel *(4)*, sedimentation may be a useful technique by which to follow changes in freeze-dried meat during low temperature storage.

One of the outstanding features of this series of experiments was the lack of any marked decrease in the level of specific ATPase activity after drying. A comparison of the results in Tables 3 and 4 shows that there is somewhat less difference in actomyosin ATPase activity of portions of the same muscle dried by different techniques than there is between the *semitendinosus* from different animals. The shelf dried samples (Table 3), although showing a marked decrease in extractable actomyosin nitrogen, retain a very high percentage (93% to 100%) of the original ATPase activity. In 3 samples the results actually show the freeze-dried product to have a higher activity than the frozen. Conceivably this finding may be due to a higher myosin N/Total N ratio in these extracts. The loss of 'solubility' without compensating loss of ATPase activity, as is also found with cod *(7)*, may be explained by current concepts of progressive denaturation *(10)*. Our results show a greater retention of activity after drying than has hitherto been reported.

When the over-all view is taken we find that there are only small differences between frozen and freeze-dried beef. There is, however, an indication that exposure to temperatures of 37° C and above for prolonged periods is to be avoided. Appreciable differences in percentage of extractable actomyosin nitrogen are noted among the different drying techniques tried, but these

differences are not consistent enough to warrant a positive statement of preference. Consequently it may be concluded, except as noted above, that all of the freeze-drying techniques are sufficiently mild to avoid gross damage to the proteins.

SUMMARY

An assessment has been made, with three samples of beef *semitendinosus*, of the changes resulting from four different freeze-drying techniques. The criteria applied were salt solubility, adenosine triphosphatase (ATPase) activity, electrophoretic mobility, and the effect of high speed centrifugation. Under the mildest set of conditions there were very small changes, but when drying was carried out for prolonged periods at 37° C or above, actomyosin solubility was decreased. ATPase activity was affected to a much smaller extent.

Acknowledgments

Electrophoretic measurements were made at the National Research Council, Ottawa. We wish to thank Dr. Dyson Rose for permission to use the apparatus and for help in carrying out this portion of the work.

The authors wish to acknowledge the technical assistance of Mrs. D. Parrott.

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BIOCHEMICAL ALTERATION OF MILK PROTEINS BY GAMMA AND ULTRAVIOLET IRRADIATION [a, b]

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The evaluation of radiation preserved foods as a source of nutrients is dependent upon adequate knowledge of the possible changes evoked in the carbohydrates, fats, proteins, and vitamins. Excellent discussions of these changes may be found in the review volume edited by Bailey *et al (2)* and it is sufficient to note here that these molecular alterations are not only subtle but difficult to detect by common laboratory techniques. As an example of the magnitude of change to be expected from radiation preservation, it has been calculated that two million roentgens (1.86 megarads [d]) could result in the rupture of approximately 0.003% of the C-H bonds present in one gram of an average food.

Effects of ionizing radiation on proteins are both interesting and typical. In general, it may be said that irradiation of proteins with gamma rays or high speed electrons will result in denaturation, degradation, polymerization or molecular rearrangement. Svedburg and Brohult *(23)* report that proteins such as hemoglobin, albumin, and hemocyanin when treated with x-rays or ultraviolet rays are split into smaller molecules as well as combined into compounds having higher molecular weight. Bellamy and co-workers *(4, 19)* have noted viscosity and solubility changes in both egg protein and casein treated with high speed electrons. Similarly, Barron and Finkelstein *(3)* noted changes in the sedimentation of bovine serum albumin following irradiation. These results indicate that the protein molecule has been split and that cross-linking and/or polymerization also occurs. Such alterations in molecular structure may correlate with the increase in free sulfhydryl groups reported by McArdle and Desrosier *(17)* following 7.40 megarad irradiation of 2% aqueous solutions of casein. In the case of ultraviolet radiation, photosensitizers such as hematoporphyrin have been shown *(10, 11)* to greatly increase the amount of protein alteration.

Wells and Osborne *(25)*, in their classical studies on the immunological properties of proteins from milk, demonstrated that chemical constitution is

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[d] One megarad equals one million rad.

related to the serological properties of the different proteins. From these investigations, one can conclude that dissimilar molecular configurations are responsible for differences in antigenic properties of proteins having similar chemical constitution. More specific proof of the fine gradations in molecular structure that may be differentiated by immunological techniques has been provided by the members of Pauling's laboratory *(20)*.

Inasmuch as gamma and ultraviolet radiation apparently alter the molecular properties of proteins, it would appear of interest to apply immunological techniques for estimating these changes. Such an approach has been suggested by Lehman and Laug *(14)*. In 1911, Doerr and Moldovan *(7)* found that ultraviolet radiation could reduce the antigenic activity of proteins. Leone *(15)* measured the antigenic response of guinea pigs actively sensitized with native or gamma-irradiated ovalbumin and challenged with the native protein. The radiation level was the equivalent of 2.1×10^5 rads. The mean sensitizing dose for the irradiated protein was 0.1–0.3 mcg protein nitrogen whereas the mean sensitizing dose for the native protein was 2.5 mcg protein nitrogen. In this case, then, the ovalbumin was made *more* antigenic than the native protein.

The present studies were undertaken to investigate further the immunological activity of proteins following gamma or ultraviolet irradiation. In order to elucidate the resultant molecular changes, a comparison of biological activity and physico-chemical properties has been undertaken. The proteins used for these studies were those occurring in raw skim milk. These proteins were selected because of the extensive information presently available for normal milk proteins and because any alteration in biological response might have valuable clinical significance.

MATERIALS AND METHODS

Source of animals and special reagents. The raw skim milk used in these experiments was received from a local dairy on the day that it was to be processed or chemically assayed. Each sample of milk was analyzed for protein nitrogen, moisture, and residual fat. The nitrogen value was uniform throughout these studies and the fat content did not exceed 0.2% at any time. The hematoporphyrin was obtained from California Corporation for Biochemical Research, Los Angeles, California. The diethyl-1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate (DDTPD) was purchased as a 5% solution in ethyl acetate from Distillation Products Industries, Rochester, New York. The albino guinea pigs were obtained from the Ziemet Bio-Farms, Madison, Wisconsin.

Gamma irradiation of the milk. Raw skim milk in screw-cap glass vials was packed and sealed in No. 2 metal cans, frozen and shipped with dry ice to the Gamma Irradiation Facility, Materials Testing Reactor, Idaho Falls, Idaho. Gamma irradiation was achieved at ambient temperature in a swimming pool type of facility utilizing spent fuel rods. The dose rate was 2.88 to 3.38 megarads per hr with an average energy of 0.75 mev. The samples were irradiated to total dosages of 0.465, 2.79, 5.58 or 9.30 megarads. The cans were then repacked in dry ice, returned to our laboratory and stored at −20° C with the non-irradiated control samples.

Concurrent radiation distillation. Samples of whole milk prepared by concurrent radiation distillation *(26)* were obtained from Massachusetts Institute of Technology. In this procedure, Cobalt-60 irradiation is achieved in a nitrogen atmosphere under reduced pressure. Volatile by-products are removed during irradiation and collected in cold traps. The radiation level was 9.30 megarads.

Ultraviolet irradiation. Raw skim milk was placed in large Petri dishes and agitated with a magnetic stirrer in a temperature-controlled room (70° F). The samples were irradiated with a Mineralite S-61 (wave length 2400–4000 Å) lamp with filter removed and with the coils 13 cm above the surface of the milk. Irradiation periods were 3 or 6

hours, both with and without the photosensitizers: hematoporphyrin (1.7 mg/100 ml milk) or DDTPD (100 mg. in ethyl acetate/100 ml milk). Following ultraviolet irradiation, the milk was restored to its original volume with water.

Gross anaphylaxis measurements. Male and female guinea pigs weighing 250–300 g were passively sensitized with 0.5 ml of anti-milk rabbit serum prepared by the method of Linder, Shaw, and Kraybill *(16)*. Forty-eight hours after sensitization, the animals were challenged with 0.5 ml of the irradiated or control milk injected into the accessory cephalic vein. The doses of total protein nitrogen ranged from 10 to 3200 γ per 0.5 ml. Severity of symptoms, time of onset of anaphylactic response and time of death were recorded for each dose. The method of probit analysis *(9)* was used to calculate the mean lethal shocking dose (LD_{50}) in terms of mcg of protein nitrogen, using the number of anaphylactic mortalities at each dose level.

Quantitative electrophoretic determinations. Since the preponderance of casein in the milk protein tends to mask the whey proteins during electrophoresis, the casein and whey proteins were separated according to the method of Hipp et al *(12)*. The casein was redissolved to a concentration of 6 mg % nitrogen and the whey to a concentration of 12 mg %; 0.06 ml of each were used for electrophoresis. Paper electrophoretic patterns were obtained using the hanging strip method of Durrum *(8)* and Block, Durrum and Zweig *(5)*. Electrophoresis was performed with a current of 6 ma for 16 hr, using veronal buffer, pH 8.6, ionic strength 0.075. A recorder-integrator was employed to convert the color density pattern into a concentration curve and to measure the area under each peak.

Viscosity measurements. Viscosity measurements were made according to the method of McGoury and Mark *(18)* at a temperature of 25° C.

Sulfhydryl and disulfide bonds. The sulfhydryl content of the milk proteins was rapidly determined by the dead-stop, end point titration procedure outlined by Larson and Jenness *(13)*. Results were expressed as mg of cysteine hydrochloride per ml. The methyl mercaptan concentration was assayed by the micro-method of Sliwinski and Doty *(22)*.

Due to the micro nature of the changes in protein structure brought about by ionizing radiations, application of a combination histochemical-paper strip chromatographic technique adapted from Thompson *et al (24)* appeared promising. The principle of this method is as follows: filter paper strips were impregnated with dialyzed or non-dialyzed milk protein solutions, dried and stained with dinitrofluorobenzene *(21)*. The DNFB reacts with free amino groups of protein, with sulfhydryl groups, and with the hydroxyl group of tyrosine to yield colored reaction products (except with tyrosine). The color of the products may be intensified by diazotization of the nitro group in the 4-position and subsequent coupling with a substituted naphthol. Blocking of any two of the 3 groups will demonstrate the significance of the third. The disulfide linkages are determined by reduction with HCN and the procedures for sulfhydryl groups applied.

RESULTS AND DISCUSSION

The mean lethal shocking doses (LD_{50}) for milk-sensitized guinea pigs challenged with gamma-irradiated milk are presented in Table 1. It may be readily seen that radiation dosages of 0.475 or 2.79 megarads had little effect upon the allergenic properties of skim milk protein. In the case of milk treated with 5.58 megarads, the LD_{50} value was approximately nine times as great as with the control milk ($602 \pm 270\,\gamma$ versus $69 \pm 16\,\gamma$ protein nitrogen). The milk given a 9.30 megarad dose yielded an LD_{50} of $1{,}157 \pm 422\ \gamma$ protein nitrogen compared to the control value of $37 \pm 4\ \gamma$ protein nitrogen. It is thus apparent that about 5.58 megarads of gamma irradiation is required to effect of a molecular alteration sufficient to decrease markedly the biological response to the treated protein.

Concurrent radiation distillation using a Cobalt-60 source yielded a product having an LD_{50} value of $131 \pm 23\ \gamma$ protein nitrogen compared to its control of $25 \pm 4\ \gamma$ protein nitrogen. This value is significantly lower than that obtained with the 9.30 megarads from the spent fuel element source. In

TABLE 1

Mean lethal shocking dosé (LD_{50}) for milk-sensitized guinea pigs challenged with gamma irradiated and raw skim milk

Series	Radiation dose 10^6 rads	Number of guinea pigs	LD_{50} γ protein N	Approx. ratio control vs. irrad.
1	0	66	36 ± 7	
	0.475	66	29 ± 4	1 : 0.8
2	0	77	32 ± 4	
	2.79	87	64 ± 11	1 : 2
3	0	64	69 ± 16 [1]	
	5.58	50	602 ± 270	1 : 9
4	0	60	37 ± 4	
	9.30	55	1,157 ± 422	1 :31
Values for concurrent radiation distilled whole milk				
5	0	68	25 ± 4	
	9.30	71	131 ± 23	1 : 5

[1] Stock of antisera had lower antibody titer.

fact, the changes in the former sample would appear to be more nearly analogous to a 3.5–4.0 megarad sample prepared by the usual radiation procedure. Two explanations of this difference are possible. One is that whole milk was used for the concurrent radiation-distillation so that the fat may have exerted a protective effect upon the proteins. The second possibility is that the removal of the smaller molecular fragments by distillation prevented further interaction with the milk protein. In either case, the net effect would be decreased protein change resulting in increased anaphylactic response.

Table 2 summarizes the biological data obtained with ultraviolet irradiation of raw skim milk. It may be seen that 3-hour irradiation of the skim milk caused an 8-fold change in the biological data (228 ± 52 γ protein nitrogen versus control of 27 ± 3 γ protein nitrogen), which is roughly equivalent to the 5.58 megarad gamma-irradiated milk sample. Additional irradiation up to 6 hr did not further increase the relative LD_{50} value of the treated milk. The addition of hematoporphyrin as a photosensitizer caused a highly significant increase in the LD_{50} value to $1{,}272 \pm 299$, somewhat greater than that achieved with 9.30 megarads of gamma irradiation. The addition of DDTPD exerted little effect on the milk protein even after 6 hr of ultraviolet irradiation. From these results it would appear that, unlike

TABLE 2

Mean lethal shocking dose (LD_{50}) for milk-sensitized guinea pigs challenged with ultraviolet irradiated and raw skim milk

Treatment	Number of guinea pigs	LD_{50} γ protein N	Approx. ratio control vs. treated sample
Control	55	27 ± 3	
3 hour	53	228 ± 52	1 : 8
3 hour + hematoporphyrin	81	1,272 ± 299	1 :49
Control	55	18 ± 3 [1]	
6 hour	56	75 ± 12	1 : 4
6 hour + DDTPD	55	96 ± 20	1 : 5

[1] Stock of antisera had higher antibody titer.

the case of gamma irradiation, a maximal effect of ultraviolet light upon milk proteins was obtained within the first 3 hr of irradiation. Further change could be produced by the addition of hematoporphyrin as a photoactivator, but not by the addition of DDTPD.

The viscosity data shed further light upon the changes taking place during the irradiation of milk protein. The data obtained with gamma-irradiated milk is presented in Table 3. It may be seen that a gradual increase in

TABLE 3

Influence of gamma irradiation on the viscosity of raw skim milk

Dosage (megarad)	Viscosity (centipoise)
0	1.498
0.465	1.533
2.79	1.574
5.58	1.574
9.30	1.715

viscosity from 1.498 to 1.574 centipoises occurred with increasing dosage up to 5.58 megarads. Between 5.58 and 9.30 megarads the viscosity changed markedly, from 1.574 to 1.715 centipoises. It is possible that these changes might reflect an increasing molecular size, particularly between 5.58 and 9.30 megarads. This polymerization might, in turn, explain the dramatic decrease in biologic response observed between the latter two radiation levels. The viscosity from 1.498 to 1.574 centipoises occurred with increased dosage

TABLE 4

Effect of sensitizers on the viscosity of ultraviolet irradiated skim milk (All values expressed in centipoises)

Dosage	No sensitizer	Hematoporphyrin	DDTPD
Control	1.377	1.392	1.391
3 hour	1.380	1.347	1.406
6 hour	1.412	1.430	1.408

do not show any significant change in viscosity resulting from exposure to ultraviolet rays. This would suggest that the alteration in biologic response seen following 3 or 6 hr periods of irradiation may be due to some factor other than molecular polymerization. Even the addition of hematoporphyrin did not induce marked viscosity changes during ultraviolet treatment.

The electrophoretic patterns obtained with the casein and whey fractions from gamma-irradiated skim milk are presented in Figures 1 and 2, respectively. The data for 0.465 megarad treatment have been omitted inasmuch as the results were nearly identical to the 2.79 megarad sample. At radiation levels of 2.79 megarads and below, the only apparent change was a decrease in the casein, albumin and globulin fractions. The residue, however, continued to move through the electrophoretic field in a normal manner. At 5.58 megarads, both the α-casein and the immune globulin had totally disappeared. An essentially immobile protein component appeared in both the casein and

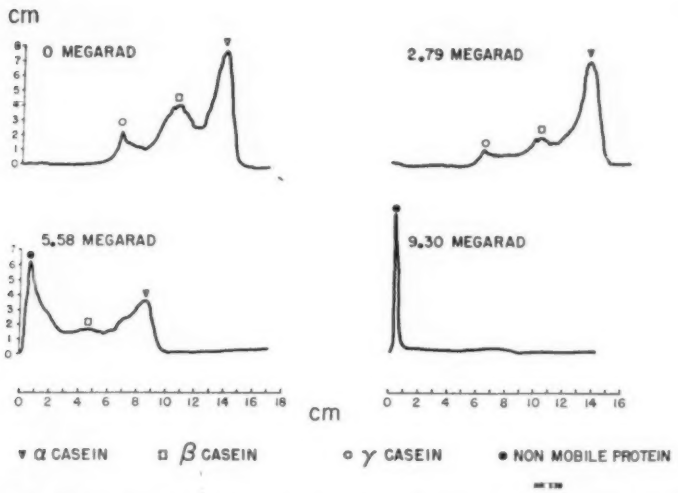

Figure 1. Effect of gamma radiation on the electrophoretic pattern of casein proteins.

whey fractions which had the effect of decreasing the mobility of the remaining β- and α-casein as well as of the α-lactalbumin and β-lactoglobulin. Following 9.30 megarad irradiation, only the immobile component was observed in either the casein or whey fractions. In connection with these findings, it was observed that at 5.58 megarads a definite "browning" occurred with the protein becoming more insoluble and gelatinous. At 9.30 megarads, the physical characteristics were so altered that solution was very difficult. It is

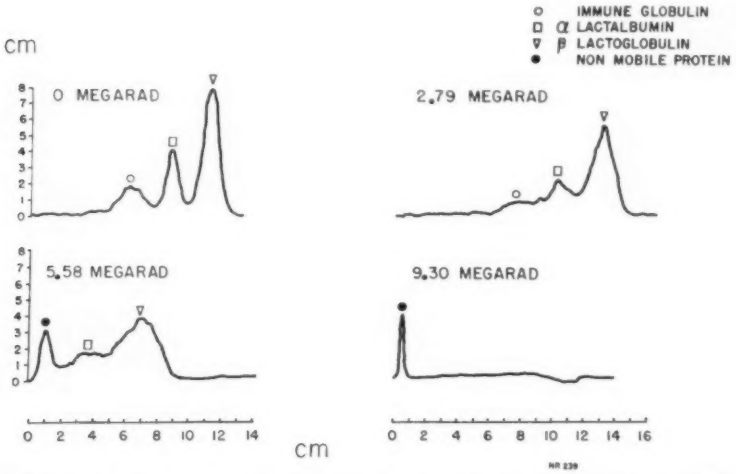

Figure 2. Effect of gamma radiation on the electrophoretic pattern of whey proteins.

interesting to note here that Brunner and Nickolas *(6)* have reported the formation of a similar non-mobile component with the casein from skim milk treated with 3.6×10^6 megarad irradiation from an accelerated electron source.

The casein and whey from ultraviolet irradiated milk was similarly investigated by means of electrophoresis. No differences between the control, 3 and 6 hr whey fractions were observed. In the case of the casein fraction, the patterns for the 3 and 6 hr treated samples were essentially the same as those obtained with the control milk except that approximately 7% of the total protein appeared as an immobile component.

The electrophoretic data suggests that degradation of protein occurs with dosages below 5.58 megarad gamma irradiation. Polymerization of both the whey and casein components occurs at 5.58 megarads and above. Ultraviolet irradiation, on the other hand, results in only a small amount of polymerization in the casein fraction with no apparent polymerization of the whey proteins.

The investigations of the sulfur compounds in the gamma and ultraviolet treated milks provide further information on the nature of the reactions taking place. As the radiation dose increased, significant increases in the sulfhydryl content were obtained with both types of radiation treatment, as shown in Tables 5 and 6. The change in the gamma-irradiated samples was most

TABLE 5

Influence of gamma irradiation on the protein sulfhydryl content of raw skim milk

Dose (megarad)	Sulfhydryl (mg $\times 10^{-2}$)
0	4.30
0.465	4.39
2.79	4.81
5.58	7.21
9.30	11.75

TABLE 6

Influence of sensitizer on sulfhydryl content of ultraviolet irradiated skim milk

Dose (hours)	Sulfhydryl (mg $\times 10^{-2}$)		
	No sensitizer	Hematoporphyrin	DDTPD
0	4.525	4.902	4.525
3	5.840	9.050	9.050
6	9.145	13.198	12.070

dramatic at 5.58 megarads and above, whereas, in the ultraviolet treated samples, the sulfhydryl content was almost linearly related to exposure time. Both the hematoporphyrin and the DDTPD accentuated the production of sulfhydryls by ultraviolet irradiation. The histochemical-chromatographic procedure also demonstrated increases in sulfhydryl content as either the gamma or ultraviolet dosage increased. Furthermore, application of this sensitive procedure to disulfide estimation indicated that an increasing gamma-irradiation dose resulted in an increase in disulfide content. Application of this method to the ultraviolet treated milk did not indicate any change

in the disulfide content, either with or without the photosensitizers. In contrast to the data by Sliwinski and Doty *(22)* using gamma-irradiated beef protein, no increase in methyl mercaptan content was observed when their method was applied to the gamma-irradiated skim milk samples.

From these analyses, it is evident that both gamma and ultraviolet irradiation result in a marked increase in sulfhydryl content of skim milk. Only gamma irradiation causes a simultaneous increase in disulfide bonds; the ultraviolet irradiation has no apparent effect. Several mechanisms might explain these findings. It is possible that methionine is converted in part to methyl mercaptan by splitting the thio-ether linkage on the protein side, thus accounting for the increased sulfhydryl content. However, this would appear improbable, at least for gamma irradiation, since no increase in methyl mercaptan was demonstrated. Methionine may also be degraded to form homocysteine. Two of the latter molecules could combine to form homocystine or an unsymmetrical disulfide could be formed between homocysteine and cysteine. This degradation-condensation scheme would account for the simultaneous appearance of sulfhydryl and disulfide linkages following gamma irradiation. It may be that the energy transfer in ultraviolet irradiation lacks the intensity to initiate such a decomposition-condensation mechanism.

The data presented here suggest that ultraviolet irradiation and low levels of gamma irradiation result in the denaturation or destruction of milk protein with concomitant appearance of sulfhydryl linkages and alteration in antigenicity. Higher levels of gamma radiation probably cause the subsequent agglomeration of the protein fragments into new protein entities having different physical properties. This agglomeration is accompanied by an increase in both disulfide and sulfhydryl bonds, suggesting that the formation of -S-S-linkages may be at least partially involved in the polymerization. Furthermore, the markedly altered biological response to the high-level gamma-irradiated milk would appear to be correlated with the formation of the new protein polymers. The lack of formation of such polymers during ultraviolet irradiation might explain the less marked change in biological response to ultraviolet irradiated milk protein.

From our data, it may be said that gamma irradiation induces greater changes in milk protein than does ultraviolet irradiation. Unfortunately, gamma irradiation also induces certain off-odor compounds, radiation flavors, and a browning of the product. Although these effects have been essentially eliminated by the concurrent-radiation-distillation technique developed by Wertheim and co-workers *(26)*, the product produced with whole milk did not exhibit as extensive alteration in biological response as did skim milk irradiated by another technique. It is possible that this decreased change in the proteins may have been due to a protective effect exerted by the fat. This possibility is presently under investigation in this laboratory. In any case, the changes in milk protein resulting from gamma irradiation would appear to be of sufficient interest to warrant clinical testing for allergenicity in the human.

SUMMARY

The alterations in skim milk proteins by gamma and ultraviolet irradiation result in a decrease in anaphylactic response when the treated milk is injected into milk-sensitized guinea pigs. Greater changes in biological response are

caused by gamma than by ultraviolet irradiation. Gamma irradiation results in dosage-related increases in viscosity, and in sulfhydryl and disulfide content. Ultraviolet irradiation causes similar increases in sulfhydryl content, but no changes in disulfide bonds. No indication of the formation of methyl mercaptan by gamma irradiation of milk was found. Electrophoresis of the casein and whey fractions shows that below 5.58 megarads, the component proteins are denatured or destroyed; at 5.58 megarads and greater, an electrophoretically immobile component appears in both fractions which, at 9.30 megarads, is the sole material present. Electrophoresis of three and six hour ultraviolet irradiated samples showed no apparent change from control milk except for the conversion of approximately 7% of the casein proteins into the electrophoretically immobile component. These results have been correlated with the biological data and a possible explanation for the results has been proposed. The need for further human clinical testing of gamma-irradiated milk has been suggested.

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PRODUCTION AND PREVENTION OF IRRADIATED ODOR IN BEEF[a]

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Over the past several years the Quartermaster Corps has been conducting a research program to determine whether radiation can supplement or replace refrigeration as a means of food preservation. With beef, the dose necessary for sterilization is responsible for what has been widely characterized as an "irradiated" or "wet dog" odor *(10, 15)*. Until recently there has been no general agreement regarding the component or components in beef from which the odor arises *(1, 3, 16)*. Similarly, there is no agreement regarding the nature of the components responsible for the odor *(1, 5, 12)*.

The intent of this study was to determine the component or components of beef from which the irradiated odor arises, the chemical nature of the odor, and a means of preventing its production. The approach was to fractionate the aqueous extract produced by refluxing the beef previous to irradiation. Characterization of the implicated fraction could then be pursued.

EXPERIMENTAL

Preparation of fractions from beef. Twelve hundred grams of defatted beef chuck was diced and refluxed overnight with 1200 ml of a 10% saline solution. The extract was collected by filtration, concentrated to 250 ml under reduced pressure, and dialyzed, first with 10% saline, and then with water for several days with periodic changes. The dialyzable fraction was concentrated, the non-dialyzable fraction recovered from the tubing, and both fractions lyophilized. An undialyzed fraction was also lyophilized. From 1200 g of meat, 35 g of non-volatile solids were obtained. Approximately ⅔ of the solids were non-dialyzable. In another study, the same fractionation was carried out on beef at 4° C.

Chemical analyses of the non-dialyzable fraction

a. *Nitrogen.* Protein nitrogen was determined by the Kjeldahl procedure and amino nitrogen by the Frame method *(9)*.

b. *Phosphorus.* Phosphorus was determined gravimetrically as $Mg_2P_2O_7$ according to the A.O.A.C. official methods of analysis.

c. *Ash.* Magnesium nitrate was added to the sample prior to ashing at 590° C.

d. *Quantitative amino acid analysis.* A 5 mg, moisture-free sample was hydrolyzed with 6 N HCl at 105° C for 48 hr in a sealed tube which had been flushed with nitrogen. A hydrolysate was also prepared from a sample which had been irradiated. The hydrolysates were adjusted to pH 2.2, applied to a Dowex 50-x-5 ion exchange column and chromatographed according to the procedure of Moore and Stein *(13)*. Fractions containing 2.5 ml were collected by means of a volume principle fraction collector, the tubes stoppered, refrigerated, and analyzed at convenience with ninhydrin after the method of Moore and Stein *(14)*. Identity of the peaks was confirmed by preparation and chromatography of the 2,4-dinitrophenyl derivatives from pooled fractions obtained from the column *(11)*.

e. *Bound iron.* A sample was hydrolyzed with 6 N HCl in a sealed tube at 105° C for 48 hr. The hydrolysate was placed on a IR-120 cation exchange column in the acid

[a] This paper reports research conducted by the Quartermaster Food and Container Institute for the Armed Forces and has been assigned No. 1005 in the series of papers approved for publication.

stage. The column was successively washed with de-ionized water to remove anions, with 2 N NH_3 to remove amino acids and other ampholytes, and with 3 N HCl to recover any cations present. The acidic eluant was then concentrated and an aliquot determined colorimetrically by the thiocyanate method. Corrections were made for the iron content of the HCl and NH_3.

Electrophoretic studies. The electrophoretic pattern of the sample before and after irradiation was obtained in veronal buffer at pH 8.7 and an ionic strength of 0.1.

Irradiation procedures

a. *General procedures.* Irradiation was performed in the gamma facility at Argonne National Laboratory, Lemont, Illinois. Unless otherwise stated, samples were sealed in evacuated metal cans and given a dose of 5 megarads. (A rad is defined as the absorption of 100 ergs of radiant energy in 1 g of the material being irradiated.) Samples were kept frozen at all times except during exposure in the facility. The samples were irradiated both as a 33% slurry and in the dry state.

b. *Multiple exposure.* Aqueous slurries were irradiated at from 1 to 10 megarads. After evaluation, they were dialyzed with 10% saline and with distilled water for several days employing periodic changes. The samples were then given an additional exposure to irradiation of 5 megarads.

c. *Addition of compounds prior to irradiation.* One and 2% concentrations of a number of compounds were added to the 33% slurry prior to irradiation.

d. *Removal of irradiated odor with additives.* After irradiation, sufficient compound was added to the sample to give a concentration of 1% of the total volume.

Preliminary chromatographic studies on extracts from the irradiated non-dialyzable fraction

a. *Ammonium salts.* Aqueous, irradiated and non-irradiated protein samples were extracted with methylal and the extract made alkaline with ammonia. The ammonium salts were evaporated to dryness and aliquots chromatographed on Whatman No. 1 paper with an isopropanol-water-ammonia solvent mixture (200/20/10:v/v/v). Chromatograms were then developed with a series of diagnostic color reagents.

b. *Dinitrophenyl derivatives.* Methylal extracts, obtained as described, were treated with 1-fluoro-2,4-dinitrobenzene according to the method of Levy *(11)*. The dinitrophenyl derivatives, found to be soluble in acid solution, were chromatographed on Whatman No. 1 paper employing a n-butanol-water-acetic acid system (60/25/15: v/v/v). The dinitrophenyl derivatives were also prepared by treating the sample directly with the reagent. They were then separated from the sample by dialysis prior to chromatography.

c. *Other derivatives.* Methylal extracts from aqueous, irradiated and non-irradiated protein samples were reacted with 3,5-dinitrobenzoyl chloride and also with 1-anthraquinonesulfonic acid. The 3,5-dinitrobenzoates were chromatographed on Whatman No. 1 paper employing a methanol-water-pyridine solvent mixture (160/40/8:v/v/v). The paper was then developed by spraying with a mixture of α-naphthylamine and potassium hydroxide. The 1-anthraquinonesulfonates were also chromatographed on Whatman No. 1 paper employing the n-butanol-acetic acid-water solvent system (60/15/25:v/v/v). The paper was developed by spraying with a mixture of chloroplatinic acid, potassium iodide and hydrochloric acid (Toennies reagent).

RESULTS AND DISCUSSION

Irradiation of the fractions. After irradiation of the fractions with 5 megarads, the samples were evaluated by an informal odor panel and the following conclusions were made. As compared with the non-irradiated controls, the undialyzed extract and the non-dialyzable fraction developed the irradiated odor while the dialyzable fraction developed only a slight off-odor characterized as being "grassy." The residue from the extraction also developed a slight amount of the irradiated odor. This was expected because no attempt had been made to extract the meat exhaustively. It therefore appears that the irradiated odor comes primarily from the non-dialyzable fraction. These results are summarized in Figure 1.

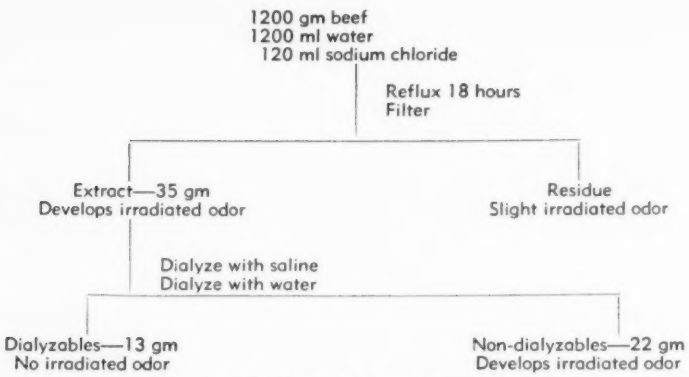

Figure 1. Irradiation of fractions from beef.

In another experiment in which the extraction was carried out at 4° C, the results obtained were only slightly altered. Compared with the non-irradiated controls, the original extract and the non-dialyzable fraction developed the irradiated odor. The odor of the dialyzable fraction seemed improved upon irradiation while the extracted residue again developed a small amount of the irradiation odor.

These results indicate that the non-dialyzable fraction is largely responsible for the development of the odor. It should be emphasized, however, that other off-odors are developed in beef upon irradiation. These may arise from interaction of this fraction with the fat or with the low molecular weight material, or from one of these fractions independently. Furthermore, the fractionation procedure may have altered or degraded the system so that additional odor producing reactions cannot occur.

Chemical and physical characterization of the non-dialyzable fraction. Since the non-dialyzable fraction was expected to consist mainly of protein, several analyses were performed for confirmative purposes. By Kjeldahl procedure the sample analyzed for 16.04% nitrogen which would be 100.25% protein assuming a conversion factor of 6.25. The amino nitrogen accounted for 13.6% of the total sample weight. The sample was found to contain 0.76% ash. Of this, 0.21% was phosphorus and 0.021% iron. Assuming a calculated average N/P ratio, approximately 5% of the sample was nucleoprotein. The detection by chromatographic methods of hypoxanthine in the sample volumes for these peaks and comparative corrected relative retention volumes served as additional evidence for the presence of nucleoproteins.

The iron was also assumed to be bound to the protein. The amount found would be expected with muscle proteins.

Quantitative amino acid analysis. The non-dialyzable fraction was irradiated and subsequently subjected to acid hydrolysis and analysis according to the Moore and Stein procedure. Compared with the non-irradiated control, several changes in ninhydrin reactive compounds were noted; however, no new compounds were found. Cysteine and methionine could no longer be detected, while the content of histidine decreased by approximately 25% as a result of irradiation of the protein. The content of serine was increased by

TABLE 1

Amino acid content, moles x 10^{-4}/gm

Peak no	Probable identity	Control	Irradiated	Peak no	Probable identity	Control	Irradiated
1	Cysteic acid	0.35	0.35	11	Glycine	2.05	1.80
2	Taurine	4.25	3.80	12	Alanine	4.40	4.60
3	Unknown	1.45	1.25	13	Unknown	2.55	2.40
4	Unknown	1.55	1.60	14	Valine	3.50	3.40
5	Hydroxy proline	1.60	1.75	15	Cysteine	0.80	0.00
6	Aspartic acid	8.00	8.20	16	Methionine	0.75	0.00
7	Threonine	9.25	9.30	17	Leucine	0.45	0.30
8	Serine	6.40	7.20	18	Aromatics	5.95	5.45
9	Proline	0.85	0.65	19	Lysine	5.15	4.85
10	Glutamic acid	2.30	2.25	20	Histidine	2.60	1.95
				21	Arginine	2.95	3.15

Tryptophan not analyzed.

12.5%. Proline and the aromatic amino acids were decreased by less than 10% and the remaining amino acids were not markedly affected (Table 1).

These observations confirm previous reports such as those by Dale and Davies *(6)* on the lability of cysteine, by Schwartz and Clark *(17)* on the lability of methionine and proline, and by Drake, Giffee, Johnson and Koenig *(7)* on the lability of histidine.

Electrophoresis of the fraction. The sample was also studied electrophoretically before and after irradiation and was found to contain at least 2 proteins, one of which was increased by irradiation, apparently at the expense of the other peak which was noticeably decreased. Whether this represents an actual formation of one protein from the other or merely a conversion to a third protein which migrates at the same rate under the conditions employed is not known.

This pattern change would not be unexpected as it has been shown that when irradiated chicken plasma protein is subjected to paper electrophoresis, spots appeared which were not present in the original protein *(4)*. Both fragmentation and polymerization of proteins by ionizing radiation have been reported. Svedberg and Brohult *(18)*, using radon as a source of alpha particles, observed that hemocyanin was split in two parts. Fernau and Pauli *(8)* noted decreases in viscosity with irradiated proteins. This decrease in viscosity, as well as bleaching in color, has been noted in the non-dialyzable fraction which is the subject of this report.

Effect of irradiation in the dry state on development of the irradiated odor. When the lyophilized sample was subjected to 5 megarads of irradiation, little or no odor was present. However, when water was added to the sample, the typical odor developed. That irradiated proteins release their odor upon addition of water has also been shown with globin, bovine plasma albumin, egg albumin, blood albumin and sodium caseinate *(2)*. The mechanism responsible for release of the odor is not understood.

Multiple exposure to radiation. A series of samples (33% slurries) was subjected to increasing doses of irradiation ranging from 1 to 10 megarads at one megarad intervals. When the odors of these samples were evaluated by an informal panel the maximum odor was judged to be produced by 3 or 4 megarads. There was an increase in odor with increasing dose from

1 to 3 megarads, and at 10 megarads the character of the odor had changed and was judged to be no more, and possibly less, offensive.

It was found that the irradiated odor could be removed by dialysis in 10% sodium chloride solution. Therefore, in order to determine whether production of odor was finite, this series of samples was exhaustively dialyzed and reirradiated with 5 megarads. Results showed that the sample previously given a dose of only one megarad produced the most odor and that the sample previously given 2 megarads produced a small amount of irradiated odor. The rest of the samples exhibited little or no odor. These data provide presumptive evidence that only a finite amount of odor is produced from this source.

Quenching of odor by additives. The irradiated protein (a 33% slurry) and the non-irradiated control were treated with a number of reagents to determine whether the odor could be quenched. Of those employed, only mercuric acetate, sodium p-chloromercuribenzoate, n-ethyl maleimide and sodium hydroxide decreased the odor. Hydrochloric acid, ninhydrin and 2,4-dinitrophenylhydrazine did not quench the odor. When cysteine was added to the irradiated sample which had been made odorless with sodium p-chloromercuribenzoate, part of the original odor returned. These data suggest that compounds with active hydrogens, probably sulfur containing, were responsible for the odor.

Prevention of irradiated odor formation. A number of compounds were added to the protein slurries prior to irradiation in an attempt to learn whether the production of odor could be prevented or masked. Table 2

TABLE 2

Effect of additives[1] to beef protein slurries on development of irradiation odor 5 megarads

Additive	Result
Cystine	No improvement, butyl mercaptan
Cysteine	No improvement, H_2S odor
Methionine	No improvement, oxidized methional (?) odor
Ascorbic acid	Acid odor, "sour"
Maleic acid	Reduced odor, becomes "tallowy"
Hydroquinone	Reduced odor
Sodium p-chloromercuribenzoate	Odor removed except for slight background
n-Ethyl-maleimide	Odor removed except for slight background
Mercuric acetate	Odor removed except for slight background

[1] 1 and 2% of the total volume.

summarizes the additives studied and the results which were observed. No apparent differences were observed if the additive concentration was raised from 1 to 2% of the total volume. It therefore seems unlikely that further increases in the additive concentration would measurably affect the results obtained under the conditions employed. It cannot be determined from these data whether the effective compounds actually protected the sample from irradiation damage or merely combined with the odor compounds as they were formed.

Preliminary chromatographic studies. Aliquots of an ammonium salt mixture prepared from a methylal extract of the irradiated non-dialyzable

fraction were chromatographed and treated with diagnostic color reagents. The irradiated sample did not give a positive diazotized sulfanilic acid reaction indicating the absence of aromatic amines and phenols. The 2,6-dichloroquinone chloroimide test for thiol imidazoles was also negative. The aroyl glycine test for substituted hippuric acids and related compounds gave a faint red spot at R_f 0.20 with the irradiated sample only. The ninhydrin test was negative. Chromatographic studies on preparations obtained by treatment of the sample with 1-fluoro-2,4-dinitrobenzene, 3,5-dinitrobenzoyl chloride and 1-anthraquinonesulfonic acid demonstrated that irradiation has altered the protein in such a manner as to make available for reaction at least two additional compounds which were not available in the original sample. Efforts to isolate and identify these compounds are in progress.

SUMMARY

The irradiation odor of beef has been shown to arise chiefly from a water soluble, non-dialyzable fraction. This fraction is a mixture of at least two electrophoretically separable proteins. It appears that the odor is associated with sulfhydryl or closely related compounds since cysteine and methionine could no longer be detected in the protein after irradiation and since the odor is quenched by sulfhydryl reagents. The protein was found to contain small amounts of iron and phosphorus.

It was shown that the irradiated odor was not released until water was added to the protein irradiated in the dry state. Evidence is presented that only a finite amount of odor can be produced from the water-soluble protein fraction.

Paper chromatographic studies have demonstrated that reagents such as 1-anthraquinonesulfonic acid, silver nitrate-bromphenol blue, 1-fluoro-2,4-dinitrobenzene, 3,5-dinitrobenzoyl chloride, and platinum iodide formed derivatives with compounds present in the irradiated protein but not in the original sample.

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EXAMINATION OF LEMON OIL BY GAS-LIQUID CHROMATOGRAPHY. II. THE HYDROCARBON FRACTION[a]

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There have been considerable improvements in the technique of gas-liquid chromatography (GLC) since the first paper in this series appeared *(2)*. New detectors employing radically different cell design and research in the area of new liquid phases have contributed to the vast improvement of this analytical method.

While it was possible to demonstrate the presence of only five distinct peaks in whole lemon oil in the first publication of this series *(2)*, improved detector response, and several new stationary phases have altered this situation considerably. In early 1958 Lipsky and Landowne *(9)* reported the use of two partition agents that were especially effective for the separation of unsaturated fatty acids: LAC-1-R296, the adipate polyester of diethylene glycol, and LAC-2-R446, adipate polyester of diethylene glycol partially cross-linked with pentaerythritol.[b] Use of these two stationary phases affords a rather extensive separation of the components present in the terpene fraction of lemon oil, and indicates that there are at least twelve components present (Table 1). In addition to the above two liquid phases, a third phase composed

[a] Supported in part by a grant from the Sunkist Growers, Inc., Los Angeles, California.
[b] Prepared by Cambridge Industries Company, Inc., 101 Potter Street, Cambridge 42, Mass.

TABLE 1

Relative retention volumes of the components in the hydrocarbon fraction of lemon oil. Stationary phase: LAC-2-R446[1] (α-Pinene = 1.00)

	$V_R°/V_R°$			
Peak	Unknown	Known	Compound	Per cent Composition[2]
1	0.380	.....	.....	0.04
2	0.504	.....	.....	0.03
3	0.732	.....	.....	0.02
4	1.00	1.00	α-Pinene	2.52
5	1.22	.....	.....	0.05
6	1.37	1.37	Camphene	0.08
7	1.67	1.67	β-Pinene	20.69
8	1.98	1.96	Myrcene	1.28
9	2.35	2.36	Terpinolene	0.14
10	2.60	2.64	D-Limonene (β-Phellandrene)	66.59
11	3.26	3.28	γ-Terpinene	6.88
12	3.99	4.01	p-Cymene	1.68

[1] Temperature 100° C; helium flow rate 145 ml/min.
[2] Calculated by integration of areas under the appropriate peaks.

of methyl abietate, a diterpene acid ester, was employed in this study. It was felt that because of its isoprenoid structure, one quite analogous to the terpenes found in the lemon oil fraction, it would be an interesting stationary phase, and might provide an efficient vehicle for terpene fractionation.

EXPERIMENTAL

The broad distillation range of lemon oil (some 150° C), indicative of the wide divergence in the boiling points of its components, makes the efficient separation of these components by GLC at a fixed temperature inconvenient or virtually impossible without resorting to temperature programming or multi-stage chromatographic columns. Since this equipment was unavailable, a preliminary separation of the components present was desirable. Such a separation should be conducted under the mildest conditions, and it was decided to employ the chromatographic deterpenation procedure of Kirchner and Miller *(7)*. By this technique the lemon oil can be separated into 2 major fractions: one, the hydrocarbon fraction (the terpenes), with a boiling range of some 60° C, and the second fraction (the compounds bearing oxygen functions, e g, esters, aldehydes, ketones, etc) with a boiling range of some 80° C. This permits examination of the 2 fractions at different temperatures, the first temperature selected so as to be optimal for separating the terpenes, and the second selected so as to be the optimum for separating the oxygenated compounds.

The lemon oils examined were deterpenated by a modified Kirchner procedure *(7)* as follows:

15 g of freshly dried (heated at 125° C for 3 hr just before use) 100-mesh silicic acid were added to a solution of 10.0 g of the essential oil in 75 ml of *n*-pentane with shaking, and the mixture was allowed to stand at room temperature with occasional shaking for 2 hr. The silicic acid was filtered-off on a sintered glass crucible under reduced pressure, and the pentane fraction dried over anhydrous sodium sulfate for 1 hr. The oxygenated fraction was eluted from the adsorbent by placing the silicic acid in 75 ml of absolute ethanol, and the mixture was allowed to stand with occasional swirling at room temperature for 2 hr. Adsorbent was filtered off as above and discarded. Ethanol was dried over anhydrous sodium sulfate. When dry, pentane and ethanol solutions were each filtered, and respective solvents removed by a rotary flash evaporator under moderately high vacuum (*ca* 1.0 mm Hg) and at 30° C. The terpene fraction and the oxygenated fraction were then stored at −10° C until used.

The gas-liquid chromatographic equipment used to separate the hydrocarbons was an Aerograph model A-90-C,[c] equipped with a ¼ in o d stainless steel column; an improved 4-cell katharometer assembly was employed for detection.

Separations accomplished by means of LAC-2-R446 employed as the stationary phase were carried out using a column 9 ft in length and ¼ in o d filled with the liquid phase on a support of diatomaceous earth firebrick (Sil-O-Cel C-22, 30- to 60-mesh) in the proportion 25 g of the LAC-2-R446 to 75 g of firebrick (i e, 25% [w/w]).

Analyses made employing LAC-1-R296 as the stationary phase were conducted using a column 5 ft in length and ¼ in o d filled with the stationery phase in the amount of 25% (w/w).

Separations accomplished by employing methyl abietate as the stationary phase were conducted using a column 5 ft in length and ¼ in o d filled with the liquid phase in the amount of 20% (w/w).

The exact experimental parameters of operation, i e, temperature, flow rate, etc, accompany the figures and tables. A one mv recording potentiometer was used at a chart speed of 30 in per hr.

RESULTS AND DISCUSSION

Examination of the terpene fractions from several cold-pressed California lemon oils revealed that there are between 10 and 12 distinct peaks in the gas-liquid chromatograms when separated on a LAC-2-R446 column. A

[c] Manufactured by Wilkins Instrument and Research, Inc., Walnut Creek, California.

typical chromatogram is presented in Figure 1. By determining the corrected retention volumes (V_R°) *(1)* of known terpenes and comparing these with those of the unknown peaks, a large number of the terpenes present were tentatively identified. These results were confirmed by an enrichment procedure in which known terpenes were added one at a time to fresh portions of the terpene fraction and re-examined by GLC.

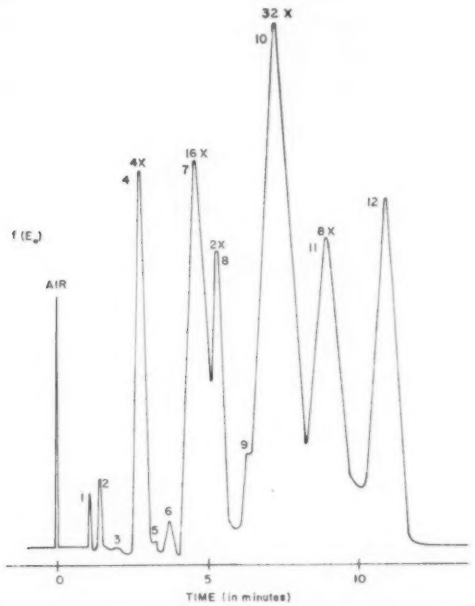

Figure 1. Chromatogram of the terpene fraction of California lemon oil. Sample size: 10 mcl; temperature: 100° C; helium flow rate: 145 ml/min; stationary phase: LAC-2-R446 on a support of Sil-O-Cel C-22 (30–60 mesh), 25% by weight; stainless steel column 10 feet in length by ¼-inch o d; 1 mv recording potentiometer; chart speed: 30 in./hr. For identification of peaks see Table 1.

There was excellent correlation between relative corrected retention volumes of unknown terpenes and those of known compounds. In no case did the difference between relative corrected retention volumes of known compounds differ from relative corrected retention volumes of the unknowns by more than 1.5% (Table 1).

When the terpene fraction was examined by means of a liquid phase consisting of LAC-1-R296, 11 distinct peaks were found. Table 2 shows that corrected relative retention volumes for the unknowns agree well with corrected relative retention volumes of the knowns. Attention may be directed to the case of peaks 10 and 11, where corrected relative retention volumes differ from those of the knowns by approximately 6%. Both James *(6)* and Bernhard *(3)* have noted that relative retention volumes must differ by approximately 20% for complete separation of zones (peaks); a difference

TABLE 2

Relative retention volumes of the components in the hydrocarbon fraction of lemon oil. Stationary phase: LAC-1-R296[1]

(α-Pinene = 1.00)

	$V_R°/V_R°$		
Peak	Unknown	Known	Compound
1	0.267	.....	.....
2	0.419	.....	.....
3	0.619	.....	.....
4	0.735	.....	.....
5	1.00	1.00	α-Pinene
6	1.43	1.46	Camphene
7	1.72	1.70	β-Pinene
8	2.03	2.10	Myrcene
9	2.85	2.84	β-Phellandrene
		2.93	and/or D-Limonene
10	3.48	3.51	Terpinolene
		3.69	γ-Terpinene
11	4.34	4.59	*p*-Cymene

[1] Temperature 100° C; helium flow rate 145 ml/min.

of 10 to 15% will show 2 united peaks; and differences of less than 7% will show a single peak. Thus it would be difficult, if not impossible, on the basis of this series of experiments, to determine whether or not these 2 components were actually present. However, data supplied by use of 2 other stationary phases lend credence to the observation that these 2 compounds are indeed present.

Examination of the terpene fraction by means of a methyl abietate column revealed the presence of 8 distinct peaks or zones. Corrected relative retention volumes for these peaks and comparitive corrected relative retention volumes for known terpenes are presented in Table 3. In this instance the corrected relative retention volume for *p*-cymene differs from that of peak 6 by 8% and from peak 7 by some 7%. It does not appear possible to resolve this ambiguity as to the proper assignment of the position of *p*-cymene on the basis of per cent composition of the 2 peaks, in this case 6 and 7, since the accuracy of the area integration method will not permit this type of determination. At best, reproducibility of per cent composition is in the nature of from 2 to 4% *(4)*. Liberti and Cartoni *(8)* have reported the presence of *p*-cymene in Italian lemon oil, and have found that it is coincident with the γ-terpinene peak when examined on a column with a stationary phase consisting of tricresyl phosphate.

We have not been able to separate β-phellandrene from D-limonene by GLC on each of the 3 liquid phases examined in this study. This fact has been confirmed by Mirov and Stanley *(10)* in their work on the terpenes in *Pinus*. Thus it is a moot point as to whether some β-phellandrene is indeed present in the hydrocarbon fraction of California lemon oil. It may be noted that β-phellandrene has been identified by workers at Schimmel and Co. *(5)* as early as 1897, and by Gildemeister and Muller *(5)* as being present in Italian lemon oil. The latter *(5)* have also identified γ-terpinene in Italian lemon oil.

Presence of a "trace" of terpinolene was reported by Liberti and Cartoni *(8)* to be present in Italian lemon oil. This, too, is in agreement with our findings (Table 1).

TABLE 3

Relative retention volumes of the components in the hydrocarbon fraction of lemon oil. Stationary phase: methyl abietate[1] (α-Pinene = 1.00)

	$V_R°/V_R°$		
Peak	Unknown	Known	Compound
1	0.921	.....	.....
2	1.00	1.00	α-Pinene
3	1.20	1.22	Camphene
4	1.44	1.44	Myrcene
		1.47	β-Pinene
5	1.78	1.81	Terpinolene
6	2.10	2.13	D-Limonene
		2.16	β-Phellandrene
7	2.46	2.27	p-Cymene
		2.51	γ-Terpinene
8	2.90	.....	.....

[1] Temperature 125° C; helium flow rate 110 ml/min.

We have been unable to isolate the sesquiterpenes bisabolene or cadinene, both of which have been reported to be present in oil of lemon *(5)*, by the methods described herein; and no peak corresponding to either hydrocarbon has been detected. On distillation of large volumes of lemon oil, Bernhard *(4)* has isolated a fraction which on treatment with hydrogen chloride gives a derivative that corresponds to the trihydrochloride of bisabolene. The physical properties are as follows: mp 79–81° C (corr); Lit. *(5)* 79–80°; per cent composition calc'd.: C = 57.4; H = 8.69; Cl = 33.9. Found: C = 57.5; H = 8.68. Cl = 33.8. Apparently, these 2 sesquiterpenes are present in extremely small quantities in lemon oil. More recently Bohnsack *(5)* has confirmed the occurrence of bisabolene and cadinene in Italian lemon oil.

SUMMARY

The hydrocarbon fraction (terpenes) of California cold-pressed lemon oil was examined by means of gas-liquid chromatography and a number of the components present were tentatively identified. α-pinene, camphene, β-pinene, myrcene, D-limonene, terpinolene, γ-terpinene, and p-cymene were the compounds identified in this fraction. A proximate analysis is presented for the composition of the terpene fraction of a typical California lemon oil.

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ANALYSIS OF COFFEE VOLATILES BY GAS CHROMATOGRAPHY

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Attempts to isolate or concentrate the components responsible for the aroma of coffee were first made more than one hundred years ago. Since that time, many investigators have contributed to this field of knowledge and several reviews *(1, 2, 3)* on the constituents of roasted coffee are available. Some of the major components such as amino acids, caffeine, trigonelline, chlorogenic acid, and tannin are non-volatile and are determined in extracts. Although a considerable effort has been made to evaluate the volatile constituents of roasted coffee, progress in this direction has been relatively slow. The chief analytical techniques used have been vacuum distillation, extraction, infrared spectroscopy and organic analysis. With the development of new instrumental methods such as mass spectrometry and gas chromatography, a new approach to this problem became evident. Indeed, Rhoades *(4)* has recently described an apparatus for the collection of coffee volatiles for gas chromatographic analysis. The present investigation involves the collection of coffee volatiles, their separation by gas-liquid chromatography and their identification by mass spectrometry.

Origin and nomenclature of volatile coffee samples. Samples considered in this paper have for convenience been designated as coffee aroma essence and dry vacuum aroma (DVA) fractions.

The coffee aroma essence fraction, largely composed of aldehydes and sulfides has a relatively low solubility in an equal amount of water and represents only a few parts per million of the original roasted coffee. It quickly evaporates at room temperature and is subject to rapid flavor and aroma deterioration when oxidized. The essence has an extremely powerful odor and is a clear, yellow-green liquid that floats on water. This essence when sufficiently diluted in the air gives true coffee aroma. It is important to note that the coffee aroma essence will vary notably with different types of coffee. It can be collected from any roasted coffee either by wetting or heating the coffee and withdrawing the volatiles. These are then condensed efficiently and completely at low temperatures in either liquid nitrogen or dry ice condensers. Invariably this essence has some moisture collected with it, but if care is exercised, it can be made relatively water-free.

The DVA acid aqueous fraction corresponds only partly to the aqueous layer cited above because of different methods of collection. DVA is about 99% water with the balance acetic acid, propionic acid, and higher molecular weight organic compounds steam distilled from the roasted coffee.

In order to concentrate the organic material present in the DVA, it was ether extracted. The product contained 25% water and will be referred to as DVAX.

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EXPERIMENTAL

Apparatus. (a). Burrell Kromo-tog with the sensing element replaced by a pair of matched thermistors to increase the sensitivity. (b). Perkin-Elmer Vapor Fractometer, 154B. Helium was used as the inert carrier gas. Both chromatographic units were operated at 25° C for the columns and the thermal conductivity cells. In one case, the less volatile DVAX was chromatographed at 100° C.

Column packings. (a). Perkin-Elmer column packing "C"-silicone oil on celite. (b). 15% of a 1:1 mixture of ethylene carbonate and propylene carbonate (Eastman) on 40- to 60-mesh C-22 firebrick. (c). Perkin-Elmer column packing "K"-Carbowax 1500 on Celite. (d). 20% tetraethyleneglycol dimethyl ether (Eastman) on 40- to 60-mesh C-22 firebrick. The C-22 firebrick was treated with aqua regia *(5)* prior to its use as the inert solid support. Column packings (a) and (d) were selected to give chromatograms that were applicable to fraction collecting for mass spectrometry. The most useful packing for analytical work proved to be (b), since it resolved the low boiling components of the coffee aroma essence very efficiently. Packing (d) was used for the DVAX.

Preparation of samples for gas chromatography. The coffee aroma essence fraction, which was relatively water-free, was chromatographed directly. The DVAX was vacuum distilled and divided into 7 cuts and a residue. Analysis by gas chromatography and mass spectrometry indicated that 60% of the sample contained water, acetic and propionic acid in the proportions 25, 30 and 5%, respectively. The final distillate cut of the DVAX fraction (b.p. 90° C/1 mm) was chromatographed using column packing (d). As each peak emerged from the column, its odor was noted.

All fractions to be used for mass spectrometry were collected in U-tubes in a liquid nitrogen bath.

RESULTS

Gas chromatography of coffee aroma essence. This resulted in 10 cuts that were suitable for further analysis by mass spectrometry. Column packing (a) was used

TABLE 1

Analysis of a coffee aroma essence

	wt %
Acetaldehyde	19.9
Acetone	18.7
Diacetyl	7.5
n-Valeraldehyde	7.3
2-Methylbutyraldehyde	6.8
3-Methylbutyraldehyde	5.0
Methyl furan	4.7
Propionaldehyde	4.5
Methyl formate	4.0
Carbon dioxide	3.8
Furan	3.2
Isobutyraldehyde	3.0
Pentadiene	3.0
Methyl ethyl ketone	2.3
C_4 - C_7 paraffins and olefins	2.0
Methyl acetate	1.7
Dimethyl sulfide	1.0
n-Butyraldehyde	0.7
Ethyl formate	0.3
Carbon disulfide	0.2
Methyl alcohol	0.2
Methyl mercaptan	0.1
Thiophene	0.1
	100.0

to make this separation. Nineteen components were identified ranging from acetaldehyde (b.p. 20° C) to n-valeraldehyde (b.p. 103° C). Several components were added to this list by chromatographing the sample on column packing (b). The material which eluted prior to furan was trapped and submitted to mass spectrometry. Table 1 presents the quantitative results of this analysis. It should be noted that the coffee aroma essence represents about 200 ppm of the roasted coffee bean.

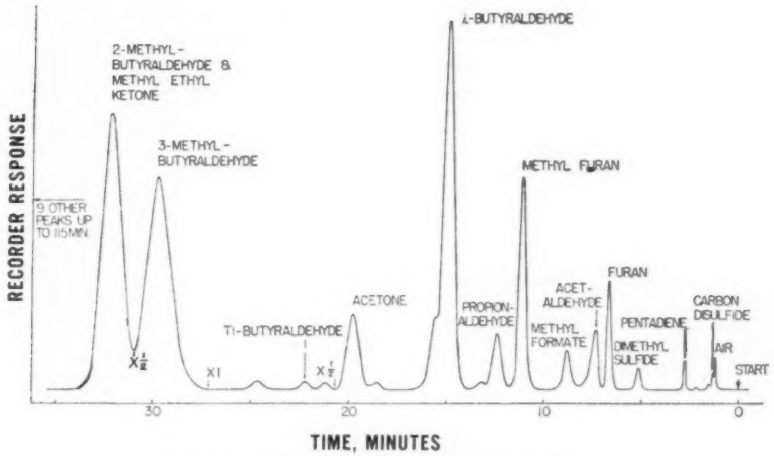

Figure 1. Chromatogram of coffee aroma essence.

A chromatogram of the essence is indicated in Figure 1. A twelve-foot column containing the ethylene carbonate-propylene carbonate packing (b) and operating at room temperature was used for this resolution. A synthetic mixture of the components from Table 1 gave an aroma which was similar to the original natural essence. Comparable chromatograms were also obtained from this synthetic mixture. The compounds identified by mass spectrometry were checked with pure samples on the chromatograph by their elution volumes.

Gas chromatography of DVAX. This was chromatographed at 100° C using column packing (c). Nine cuts were made and analyzed by mass spectrometry which resulted in the identification of more than 15 compounds. The compounds of the DVAX fraction are listed in Table 2. This DVAX represents 0.1% of the roasted coffee bean.

Examination of the odors of the peaks of the DVAX fraction as they emerged from the chromatographic column indicated the presence of pungent organic acids as well as pyridine-like odors.

TABLE 2

Analysis of DVAX coffee volatiles

Methyl formate	Methyl ethyl ketone
Ethyl formate	C_6 Ketones
Methyl acetate	Ethyl alcohol
Formic acid	n-Butyraldehyde
Acetic acid (30%)	i-Butyraldehyde
Propionic acid (5%)	C_6 Aldehydes
Water (25%)	Furfural
Acetone	Furfural derivatives
C_6 Esters	

DISCUSSION

Fugitive coffee aroma can be recovered as a tangible liquid essence at about 200 ppm from roasted coffee. The constituents that are responsible for the aroma of roasted coffee can be conveniently analyzed by gas-liquid chromatography and mass spectrometry. The very volatile coffee aroma essence fraction has proven to be more susceptible to quantitative analysis than the less volatile DVAX fraction. The latter is of higher molecular weight and probably contains more components than the essence fraction.

The odor of the emerging peaks of the essence chromatogram indicates the wide variety of odors which contribute to the organoleptic quality of coffee aroma.

The gas chromatographic work with coffee volatiles has aided in the selection of stationary phases suitable for the analysis of these complex mixtures. A packing composed of ethylene carbonate and propylene carbonate on aqua regia treated C-22 firebrick appears to be the most useful chromatographic column for analyzing the coffee aroma essence. This column will resolve the isomeric methyl butyraldehydes.

Current developments in gas chromatography using ionization detectors and capillary columns *(6)* should prove quite useful in furthering the analysis of coffee aroma. The study of volatiles of roasted coffee from different sources as well as instant coffees can be accomplished by these analytical methods. The gas chromatographic technique also presents an approach to quality control for raw materials and production in the coffee industry.

SUMMARY

Coffee aroma essence collected from commercial percolator vent gases has been separated and analyzed by gas chromatography and mass spectrometry. Dry vacuum aroma ether extracts have also been prepared from R & G coffee. More than thirty volatile components of roasted coffee which contribute to this aroma and flavor have been isolated and identified in this study. These compounds include mercaptans, aldehydes, ketones, esters, acids, and heterocyclics. It is of particular interest to note the presence of low molecular weight hydrocarbons in coffee aroma essence.

Acknowledgment

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NITROGEN COMPOUNDS OF CABBAGE. I. THE RELATION OF THE NON-PROTEIN TO THE TOTAL NITROGEN WITH SPECIAL REFERENCE TO THE ESSENTIAL AMINO ACIDS

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In addition to its nutritive value, antibacterial, antifungal, antithyroidal and antiulcer properties have been ascribed to the fresh juice of cabbage. Although the antitbiotic, effective against Gram-positive and Gram-negative bacteria, has been described to be of a carbohydrate nature *(9)*, and the antifungal and goitrogenic substances to be polysulfides *(19, 20)*, none of these factors has as yet been clearly characterized. Moreover, there have been few comprehensive studies of any of the several classes of nitrogen compounds present in cabbage.

Perhaps the greatest concentration of interest centered on the nitrogen constituents of cabbage has revolved about the indole derivatives by researchers on natural plant growth (auxins) substances. Holley *et al (16)* first reported that the major indole was probably 3-indoleacetic acid. Neither Jones and Taylor *(21)*, nor Housley and Bentley *(17)* found any indication of the presence of this acid, although the latter workers admitted it may have been destroyed on extraction. Jones *et al (22)* isolated growth-promoting 3-indoleacetonitrile. While Denffer *et al (8)* and Fischer *(13)* claimed the presence of 3-indolealdehyde in cabbage based on paper chromatograms, it was Jones and Taylor *(21)* who recently isolated and identified this substance as well as 3-indolecarboxylic acid from both maturing and fully grown plants.

The thiocyanate and isothiocyanate content of cabbage has been investigated. The equivalent of 0.1% KCNS in the fresh tissue has been reported *(36)* while Gemeinhardt *(14)* found 302 γ % expressed as HCNS in freshly expressed juice. Jensen *et al (18)* found by paper chromatography that the allyl substituent is the predominant isothiocyanate in both white and red cabbage. In the former case this compound has been unequivocally confirmed *(28)*. The minor volatile compounds of this class differed. 3-Butenyl- *(18, 28)*, sec-butyl- *(28)* and probably sec-butenyl- *(18)* isothiocyanates were observed in two separate varieties of white cabbage while the benzyl derivatives were probably a constituent of the red variety *(18)*. Although uncharacterized polysulfides and a carbohydrate in cabbage have been credited with the antifungal and antibacterial properties, respectively, various isothiocyanates have been known for some time to exert inhibitory effects against these classes of organisms. The antithyroid compound 5-vinyl-2-thio-oxazolidone was isolated from cabbage seeds (1.5 g/kg) by Astwood *et al (3)* and

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recently, some evidence has been presented to indicate its possible presence in fresh cabbage leaf *(2)*.

Most of the known vitamins and vitamin-like substances in cabbage have been assayed. Table 1 summarizes the findings on the nitrogen-containing vitamins.

TABLE 1

Vitamin	Source	Quantity	Reference
		γ per gram fresh weight	
Thiamin	White cabbage	0.60–0.70	*(38, 42)*
	Red cabbage	1.00–1.50	*(38)*
Riboflavin	Cabbage	0.27	*(40)*
Pantothenic acid	Cabbage	1.2	*(40)*
Niacin	Cabbage	4–11	*(4)*
Folic acid	Cabbage	0.9 (free) 1.9 (total)	*(26)*
Folinic acid	Cabbage	0.18 (free) 0.41 (total)	*(26)*

Virtually no study has been published on the purine and pyrimidine nitrogen content of cabbage. deMan and deHeus *(31)* had found the purine nitrogen to equal 4.55% of the total nitrogen.

Although few complete amino acid and protein investigations have been undertaken, the isolation and characterization of two new sulfur amino acids from the non-protein nitrogen fraction of cabbage leaf has proven to be of considerable interest. McRorie *et al (32)* isolated and identified 3-amino-3-carboxypropyldimethylsulfonium salt, an analogue of methionine. This compound has been shown to be a source of labile methyl groups in transmethylation reactions and is able to substitute for methionine in a variety of organisms and enzymic systems. It apparently is not identical with the antiulcer factor reported in cabbage *(1)*. Although S-methyl-L-cysteine sulfoxide isolated from cabbage by Synge and Wood *(43)* is not a compound whose metabolic significance in the plant is easily understood, it represents a very substantial proportion of the organic sulfur of cabbage *(34, 43)*. Moreover, it has been shown that dimethyl sulfide and H_2S, the main substances accounting for the disagreeable odor of cooked cabbage, arise from this newly discovered sulfoxide *(6, 15)*.

In some of the early recorded work, small quantities of choline and betaine has been isolated from cabbage *(45)*. Rau and Ranganathan *(37)* had made a study of various nitrogen fractions of the non-protein nitrogen. Some of their findings would probably now require re-examination in the light of development of improved analytical methods. Such re-examination of earlier findings applies to the work of Schryver *et al (39)* who had reported hydroxylysine in cabbage leaf protein (1.55% hydroxylysine N of the total leaf albumin nitrogen). In other work of this period, Davies *(7)* had extracted and studied the protoplasmic proteins of cabbage leaf by the chemical methods afforded at the time. Employing microbiological methods Lyman and Kuiken *(29)* and Edwards, *et al (12)* have assayed the essential amino acids in whole cabbage. Very recently, Majunder *et al (30)* have reported on the amino acid composition of cabbage employing quantitative paper chromatography. In all of this work, however, no effort was made to

distinguish between the protein and non-protein fractions. In the analyses by earlier workers *(37, 39)*, the presence of hydroxyproline was suggested; presently, Majumder *et al (30)* also indicate the existence in cabbage of this uncommon amino acid. Some work on the enzymes of cabbage has shown the presence of peptidase activities toward leucyl and glycyl peptides *(5, 10)* but the identity of those peptides actually present in cabbage has not been disclosed.

The reported isolation of the alkaloid narcotine from cabbage *(27)* probably represents a most unusual find. However, up to the present time no published work has corroborated the presence of this compound in cabbage.

Cabbage is an important food item in the American diet. Nearly a million and a half tons are produced yearly for food, of which about a quarter of a million tons are processed, mainly as canned sauerkraut. The million and a quarter tons grown for fresh market are used for cooked cabbage, coleslaw, pepper hash, salad mixes and uncooked sauerkraut.

The objective of the present work was to study the distribution of total nitrogen and Van Slyke amino nitrogen in the protein and non-protein fractions of cabbage and to determine the amounts of the "ten" essential amino acids in whole cabbage and in both fractions after separation by electrodialysis and other methods.

This basic information is necessary to the determination of the role of the various nitrogen compounds in the deteriorative changes which occur during processing and storage of vegetable products.

EXPERIMENTAL PROCEDURES

Preparation of material. Wisconsin and Danish strains of Copenhagen Market cabbage were harvested from nearby fields in lower Bucks County, Pennsylvania. They were harvested in separate years during different periods of the year at market maturity by selecting, at random, 24 heads of uniform size and shape from large fields of this vegetable.

The coarse outer leaves were removed and the heads were thoroughly rinsed to remove any spray residue or other foreign matter. Each head was quartered and most of the core was removed in the form of a wedge after which the leafy quarters were coarse chopped and the chopped material from all 24 heads was mixed. A portion of this mixture was removed for alcohol extraction and the remainder frozen and stored at −20°C. When needed for further experimental determinations, the frozen cabbage was ground with dry ice to about 2 mm in a cold Wiley Mill and the dry ice allowed to evaporate from the frozen material. For electrodialysis studies a slurry made from this material with distilled water was circulated several times through a cold colloid mill at 0.025 mm between plates.

Separation of non-protein from protein fraction. Electrodialysis was the principal method used for the separation of the non-proteins from the proteins of cabbage. Since this procedure often produces heating in its initial stages, it was necessary to provide adequate cooling and stirring plus voltage control to prevent this heat formation at any point in the electrodialysis cell. Figure 1 shows the cell used in these experiments. It consists of three 8½ x 8½ x 2 in lucite blocks hollowed to make 3 chambers, each holding approximately 1 liter of liquid when clamped together with parchment paper sheets as dialysis membranes. Each chamber contains a U-shaped cooling coil of thin-walled ⅜ in. diameter tin tubing through which an aqueous alcohol coolant can be circulated from a refrigerated cooling bath. The center chamber into which the cabbage slurry was placed was stirred by means of a standard laboratory stirrer equipped with a glass paddle. The cathode and anode chambers containing 2 in square platinum electrodes were stirred by employing rapidly rotating teflon coated magnetic stirring bars held in place against the polished lucite faces of the outer chambers by magnetic stirring motors. All 3 chambers were fed through stoppered holes in the tops and drained

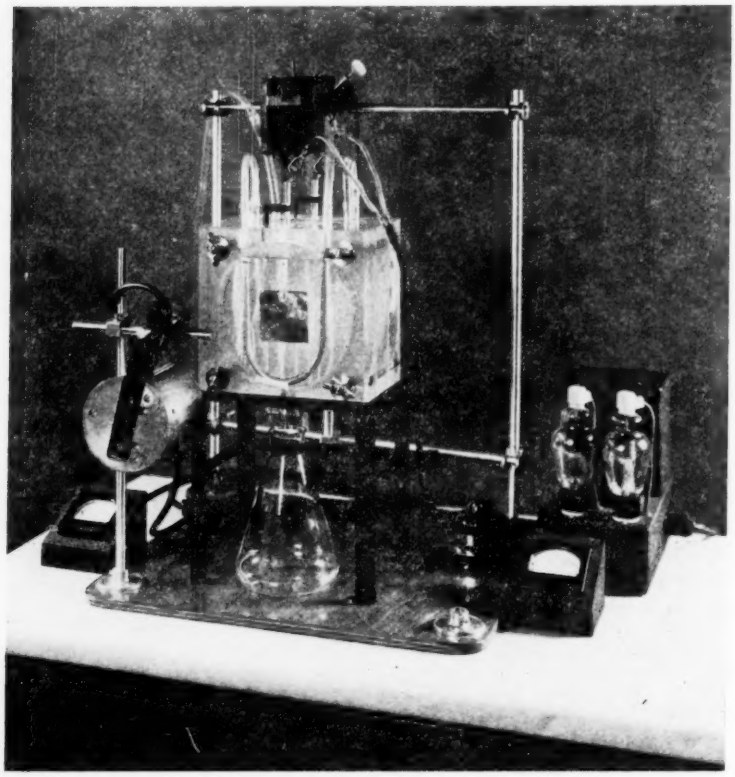

Figure 1.

through stainless steel petcocks in the bottoms of the chambers. Continuous distilled water flow could be maintained in this electrodialysis cell but in the experiments described in this paper the operation was carried out batchwise. DC voltage from 0 to a maximum of 250 v at 500 ma was provided by a "Variac"[b] controlled power pack. The temperature of the center chamber was maintained at 3–7° C by rapid circulation of the coolant and careful application of the current during the initial stages of an experimental run. Dialysis was considered complete when the current remained below 10 ma at 250 v DC for several hours.

Approximately 15 g MFB (moisture free basis) of colloidized cabbage suspension was placed in the center compartment and dialyzed against distilled water in the anode and cathode chambers. pH values were obtained at frequent intervals. The outer cell solutions were replaced with distilled water. Electrodialysis was continued until no appreciable current flowed through the cell (5 or 6 changes). Cathode solutions were acidified with HCl after removal from the cells and stored at 3° C until all the portions were collected. The respective dialysates were combined and concentrated to a volume of approximately 150 ml by vacuum concentration at 30° C, then made to a known volume for analysis. The residue fraction was washed out of the center cell with cold distilled water and made up to 2 liters. Aliquots were removed for analysis and the remainder was dried by freeze drying.

In an alternate procedure non-protein nitrogen was also extracted with 70% aqueous ethanol by the method described in the second paper of this series *(46)*.

A supplementary method of extraction was carried out by refluxing about 15 g (MFB) of colloidized cabbage in 300 cc of water for 2 hr, followed by filtration with subsequent washing of the residue with hot water. The filtrate was concentrated as before and analyzed for Kjeldahl nitrogen, arginine, threonine, and lysine. An aliquot of the hot water extract was made to 70% alcohol concentration with absolute ethanol and the slight amount of precipitate formed was removed. Both fractions were analyzed for total nitrogen and the 3 amino acids mentioned above.

Methods of analysis. Solids determinations on all samples were made by drying the samples to constant weight in a vacuum oven at 70° C. The Kjeldahl nitrogen values were determined by the official A.O.A.C. method *(33)* using semi-micro Kjeldahl apparatus. Amino-nitrogen determinations were made by the Van Slyke method with a modified auxiliary reaction chamber as used by Doherty and Ogg *(11)*. Nitrate nitrogen was determined by the method of Jones and Underdown *(23)*.

Ten amino acids—arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, valine, threonine and tryptophan—were determined quantitatively by the microbiological method of Stokes *et al*, as modified by Kelley and Baum *(24)*. Aliquots of all fractions, either vacuum concentrated or lyophilized, were hydrolyzed in 10 cc of 10% HCl in sealed ampules for 10 hr in an autoclave at 15 lb pressure. A 2 hr hydrolysis was used for the analysis of methionine *(24)*. Alkaline hydrolysis was used for the analysis of tryptophan. Unhydrolyzed samples of the electrodialyzed cathode fractions gave unsatisfactory lysine values when the organism *Streptococcus faecalis* R was used. When *Leuconostoc mesenteroides* P 60 was tried using Difco Bacto Lysine Assay Medium,[b] consistently satisfactory results were obtained on these fractions. Electrometric titration of the lactic acid formed in this microbiological procedure is now being used in place of the colorimetric titration used in the above method *(24)*.

RESULTS AND DISCUSSION

Table 2 shows the distribution of solids, Kjeldahl nitrogen and Van Slyke amino nitrogen in different fractions of 2 strains of Copenhagen Market cabbage. In view of the nature of the raw materials the results show a remarkable area of agreement in the distribution of both solids and nitrogen in the 2 batches of cabbage used in these experiments.

It is of interest to note the high percentage of solids and nitrogen in the soluble fractions, and the close agreement between the amounts obtained by dialysis and by 70% alcohol extraction. The per cent of Van Slyke amino nitrogen follows a similar pattern. Distribution of amino N in the cathode, anode and residue fractions is that obtained under the conditions of uncontrolled pH changes which occur during the course of the dialysis. These changes will be noted later in the discussion. The recovery of amino N in the 3 fractions amounts to approximately 96% of the Van Slyke amino N of the whole cabbage. The low value of 2.4% amino N in the residue after dialysis indicates that the nitrogen of the residue (47% of the total N) is principally in the form of undialyzable polypeptitde and protein N. The present paper deals primarily with the soluble N compounds, but preliminary work indicates the protein is largely in the solid phase of the residue in the central compartment since the supernatant solution, although ninhydrin positive, gives no protein precipitate. This work will be reported in a subsequent paper on cabbage nitrogen components.

The nitrate nitrogen of cabbage is not listed with the total N in Table 2 since it is less than 0.1% as determined by the method of Jones and Underdown *(23)*.

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[b] Mention of this name does not imply endorsement of this product by the Department of Agriculture over any other similar products not named.

TABLE 2

Distribution of nitrogen and solids in cabbage fractions

Fraction	Strain	Solids		Total N mg/g (MFB)	% of total N	Van Slyke amino N mg/g (MFB)	Van Slyke amino N as % total N	Van Slyke amino N as % soluble N
		mg/g Fresh wt.	% of total solids					
Whole Cabbage	1[1]	73.6		25.4		7.75	30.5	
	2[2]	56.3		29.6		10.54	35.6	
Soluble								
Dialysate	1	48.9	66.5	14.0	55.1	6.43	25.3	45.9
	2	38.5	68.3	17.0	57.3	9.31	31.45	54.9
Alcohol extract	1	49.2	66.9	14.4	56.6	6.32	24.9	44.0
	2	40.0	71.0	17.3	58.4	10.03	33.9	59.2
Dialysate								
Cathode	1	24.5	33.3	9.35	36.8	5.26	20.7	48.1
	2	21.8	38.8	10.39	35.1	7.34	24.8	57.1
Anode	1	18.3	24.8	4.65	18.3	1.52	6.0	
	2	16.7	29.6	5.92	20.0	1.97	6.65	
Non-Dialyzable Residue	1	27.8	37.7	12.09	47.7	0.61	2.4	
	2	17.2	30.6	13.62	46.0	0.89	3.0	

[1] Strain 1 refers to Wisconsin Copenhagen cabbage.

[2] Strain 2 refers to Danish Copenhagen cabbage.

Table 3 gives the values for the 10 amino acids commonly referred to as the essential amino acids in hydrolyzed whole cabbage and in the fractions obtained by electrodialysis of a cabbage slurry. Both anode and cathode fractions were studied but all 10 amino acids listed in this table are found only in the cathode fraction under the conditions of our dialysis experiments. The table shows that most of these amino acids are quantitatively accounted for in the cathode and residue fractions as evidenced by the recovery figures in the last 2 columns. Of the 10 "essential" amino acids in cabbage, arginine is the only one present in greater amounts in the non-protein than in the protein fraction (68.8% in the cathode to 36.5% in the residue). The other 9 acids in the cathode range downward from 36.3% for histidine to 2.2% for tryptophan.

The cathode fractions were analyzed both before and after hydrolysis with 10% HCl and the differences can be noted in Table 3.

It is difficult to explain these differences particularly in the case of arginine where the amount of this amino acid in the cathode is considerable. With 83.4% of the arginine in whole cabbage recovered in the unhydrolyzed cathode compared to 68.8% in the hydrolyzed fraction it is indicated that some growth stimulant is present in the unhydrolyzed cathode. It is possible that short chain peptides passed through the membranes and acted as a stimulant to the growth of the test organism in the manner described by Klungsøyr *et al (25)*. One would expect these peptides to be split by the 10 hr autoclave hydrolysis and this appeared to be the case with the Wisconsin strain of cabbage since the total recovery of arginine in the residue and the hydrolyzed cathode was 105% as compared to the 120% recovery for the residue plus the unhydrolyzed cathode.

Recovery of lysine in the cathode fractions was more variable in the different runs than any of the other amino acids, particularly in the unhydrolyzed cathodes. When *Streptococcus faecalis* R was used as the assay organism the lysine recovery from the unhydrolyzed samples and the residue was as low as 80% of the total lysine indicated by the value in the whole cabbage. Somewhat better recovery was obtained when *Leuconostoc mesenteroides* P60 was used (85–95%).

Tryptophan was apparently partially destroyed by the dialysis treatment and the amount in the cathode was so small that accurate analysis was difficult.

Table 4 has been designed to show the total and Van Slyke amino nitrogen distribution of each of the ten amino acids in the Wisconsin strain of Copenhagen Market cabbage calculated from the values in Table 3. Table 4 also shows the relationship of each and the sum of the 10 acids to the total N and Van Slyke amino N as found for whole cabbage and for the cathode fraction.

With an average of 97.5% recovery of the essential amino acids in the cathode and residue fractions it is interesting that these acids account for 32.6% of the total N in the whole cabbage with 11.9% in the cathode after electrodialysis. 36.6% of the cathode N is due to the N of the essential amino acids. As shown in Table 2, 30.5% of the nitrogen in cabbage was determined as Van Slyke amino N and 62.3% of this N was accounted for by the 10 essential amino acids in the whole cabbage and 15.25% in the cathode. The Van Slyke amino N in the cathode essential amino acids is 4.65% of the total

TABLE 3

Microbiological assay of 10 amino acids in cathode and center cell residue of electrodialyzate and whole cabbage (Wisconsin Copenhagen)[1]

Amino acid	Whole cabbage mg/g (MFB)	Cathode				Residue hydrolyzed		Recovery %	
		Unhydrolyzed		Hydrolyzed					
		mg/g (MFB)	% of total amino acid	mg/g (MFB)	% of total amino acid	mg/g (MFB)	% of total amino acid	Cathode unhydrolyzed + residue	Cathode hydrolyzed + residue
Valine	5.7	0.9	15.8	1.1	19.3	4.7	82.5	98.3	101.8
Histidine	2.2	0.9	40.9	0.8	36.3	1.2	54.5	95.4	91.0
Arginine	9.6	8.0	83.4	6.6	68.8	3.5	36.5	119.9	105.3
Leucine	6.7	0.5	7.5	0.5	7.5	5.7	85.1	92.6	92.6
Threonine	5.1	1.3	25.5	1.2	23.5	3.7	72.5	98.0	96.0
Methionine	1.9	0.1	5.3	0.2	10.5	1.7	89.5	94.8	100.0
Lysine	7.5	0.9	12.0	1.1	14.7	5.5	73.4	85.4	88.1
Tryptophan	0.9	0.04	4.5	0.02	2.2	0.5	55.5	60.0	57.7
Isoleucine	4.6	0.5	10.9	0.9	19.6	3.9	84.8	95.7	104.4
Phenylalanine	4.9	0.51	10.4	0.38	7.8	4.2	85.7	96.1	93.5

[1] The figures in this table are the average of 2–5 replicates of at least three dialysis experiments.

TABLE 4

Distribution of total and amino nitrogen calculated for the 10 essential amino acids in whole and soluble cabbage fractions

Amino acid	Total N of amino acid as % of total nitrogen		Total N of amino acid as % of total soluble [1] nitrogen	Amino N of amino acid as % of total nitrogen		Amino N of amino acid as % of total amino nitrogen	
	Whole cabbage	Cathode fraction	Cathode fraction	Whole cabbage	Cathode fraction	Whole cabbage	Cathode fraction
Valine	2.68	0.52	0.94	2.69	0.52	8.80	1.70
Histidine	2.14	0.85	1.55	0.78	0.28	2.55	0.93
Arginine	12.12	8.37	15.20	3.04	2.09	9.95	6.85
Leucine	2.81	0.21	0.38	2.81	0.21	9.23	0.68
Threonine	2.36	0.55	1.01	2.36	0.56	7.74	1.82
Methionine	0.70	0.07	0.14	0.70	0.07	2.30	0.25
Lysine	5.67	0.83	1.51	5.67 [2]	0.83	9.25	1.35
Tryptophan	0.49	0.01	0.02	0.24	0.003	0.80	0.01
Isoleucine	1.93	0.38	0.68	1.93	0.38	6.34	1.24
Phenylalanine	1.64	0.12	0.23	1.64	0.13	5.37	0.41
Total of 10 essential	32.60	11.91	21.66	21.89	5.06	62.32	15.25

[1] Cathode and anode.

[2] Lysine yields from 80–90% of its ϵ amino N by the Van Slyke method.

N. As noted in Table 4 the Van Slyke amino N of lysine is greater than its theoretical value based on α-amino N only.

Data in Table 5 were obtained in order to compare the recovery of arginine, threonine and lysine using electrodialysis, aqueous ethanol and hot water (100° C) extractions, and to determine the values for these 3 amino acids in the Danish strain of Copenhagen Market cabbage. Our interest in the use of aqueous ethanol and hot water as extractants of the soluble nitrogen constituents of vegetables came about as a result of our chromatographic studies *(46)* in which aqueous alcohol was used as the extractant. Arginine and lysine values were lower in the alcohol extracts analyzed by the chromatographic method than in the electrodialyzed cathode fraction analyzed by the microbiological procedure. Oland and Yemm *(35)* reported difficulty in quantitatively extracting arginine from apple stem tissue with 70% aqueous alcohol and they removed about 20% more arginine by hot water extraction.

TABLE 5

Total nitrogen and amino acids in Danish Copenhagen cabbage fractions as determined on hydrolyzed extracts by microbiological assay

Sample	Kjeldahl N % (MFB)	Arginine		Threonine		Lysine	
		mg/g (MFB)	% of total arginine	mg/g (MFB)	% of total threonine	mg/g (MFB)	% of total lysine
Whole cabbage slurry	2.95	9.2		5.0		6.4	
Cathode	1.26	6.7	72.8	1.3	26.0	1.4	21.9
Anode	0.43	—	—	—	—	—	—
Residue	1.22	4.0	43.5	4.0	80.0	5.0	78.1
Recovery %	98.6		116.3		106.0		100.0
EtOH extract	1.72	6.2	67.4	1.6	32.0	0.9	14.1
Residue	1.17	4.2	45.7	3.6	72.0	5.0	78.1
Recovery %	98.0		113.1		104.0		92.2
Hot H_2O extract	1.88	6.3	68.5	1.7	34.0	1.7	26.6
Residue	0.94	3.4	39.1	2.8	56.0	4.0	62.5
Recovery %	97.3		107.6		90.0		89.1

In Table 5 all extractions were made simultaneously from identical batches of cabbage and all samples were hydrolyzed and analyzed for arginine, threonine and lysine by the microbiological method. Threonine was included in this group because previous values were in good agreement by both methods of extraction and analysis.

Total nitrogen values are included in this table for better comparison of the three extracts. It can be seen that the combined cathode and anode fractions containing 1.69% of the total N of whole cabbage and the ethanol extract containing 1.71% N are in good agreement but the hot water extract with a value of 1.88% N is about 10% higher in total N. The hot water residue, on the other hand, is about 13% lower than the average of the dialysis and alcohol residues. Total recovery of nitrogen in each extract is close to 98%.

Arginine values in the 3 extracts are in good agrement and the cathode value for the Danish strain is very close to the averaged value found for this amino acid in the Wisconsin strain.

Extraction of arginine by aqueous alcohol was apparently complete since neither dialysis nor hot water removed a significantly greater amount from this cabbage tissue.

Arginine in the residue from the Danish cabbage was higher than that found in the residue from the Wisconsin strain and again the total recovery was high (116%) of the whole cabbage. Since both the cathodes and residues and the ethanol extracts were hydrolyzed in these experiments, it is difficult to understand how a peptide could account for a more rapid growth rate of the test organism. Further study will be needed to determine the actual existence in cabbage of such a growth factor and what its nature might be.

Data in Table 5 show that both alcohol and hot water appear to extract a somewhat larger amount of threonine than electrodialysis but the total recovery of this amino acid in all 3 extracts is within the ± 10% limit of this method of assay. Likewise aqueous alcohol appears to extract less lysine than either dialysis or hot water, but at the low levels at which this amino acid occurs in the soluble fractions the differences may not be significant. Recoveries of both threonine and lysine in the residue fractions of the hot water extraction experiments are appreciably lower than those in the dialysis and alcohol extraction, but since total recoveries are close to 90% even more accurate methods of analysis will be needed to explain these differences.

In this paper we have attempted to show the relationship of the free essential amino acids to the protein-bound ones and to determine whether certain methods of extraction are more suitable than others for the separation of free and bound amino acids. In the case of the amino acids studied so far, electrodialysis, aqueous alcohol, and hot water extraction methods are equally suitable. This same statement cannot be made for certain other amino compounds present in cabbage tissue in considerable amounts. Zacharius *et al.* in the second paper of this series *(46)*, have identified and estimated an additional 11 amino compounds and postulated the presence of others which contribute to the total nitrogen of the tissue extract. A complete nitrogen balance can be obtained only after all of these compounds are quantitatively determined.

In the electrodialysis method of separation of non-protein from protein amino compounds at least 2 substances greatly complicate the study of the nitrogen distribution in vegetable tissue. These are the amides glutamine and asparagine. Table 2 shows that all of the nitrogen can be recovered in the anode, cathode and residue fractions but the distribution is not always the same from run to run. This was shown particularly by the Danish strain in 2 different electrolytic separations. Since the recovery of 10 of the amino acids is consistent, it is probable that changes in the nitrogen distribution in the three compartments are the result of the breakdown of the amides. In particular, glutamine can be converted to pyrrolidone carboxylic acid or glutamic acid with the concurrent release of ammonia. Both acids would be expected to migrate to the anode compartment.

The pH changes that occur during electrodialysis are probably a result of the breakdown of this compound and of asparagine *(46)*. Starting with an initial pH of 5.8 in the cabbage slurry the pH of the cathode reached 10.4 within 15 min and 12.0 within 1½ hr. It dropped to pH 7.5 after 3 hr. The central cell dropped to pH 3.0 in 15 min and rose to pH 3.4 in 3 hr remaining

close to this figure throughout a run. The anode dropped as low as pH 2.0 in 15 min and rose to pH 2.7 in 3 hr. At no time could any ammonia odor be detected and it is probable that ammonium salts were formed in the dialysis cells; these could account for the pH drop in the cathode.

At present it seems possible to conclude that electrodialytic separation of the essential amino acids and possible others is practical but that alcohol extraction is better for the extraction of all the free amino compounds of cabbage tissue. For separation of protein and non-protein nitrogen fractions, electrodialysis methods should be more suitable since no coagulation of the protein compounds can occur at the low temperatures maintained under our experimental conditions.

Since no comparable data on free amino compounds in cabbage can be found in the literature it is impossible to compare our values with those of others. Majumder *et al (30)* found no measurable arginine or methionine in certain extracts which they made, but it is difficult to deny the presence of these amino acids in the free form in all of our extracts. Total values for the essential amino acids in whole cabbage have been found by Lyman and Kuiken *(29)* and Edwards *et al (12)* and our values for these same acids are in good agreement with their findings.

SUMMARY

Non-protein nitrogen compounds of two strains of Copenhagen Market cabbage have been separated from the protein constituents by electrodialysis, 70% aqueous alcohol extraction and hot water extraction. Whole cabbage, extracts and residues have been analyzed for total solids, total nitrogen and α-amino nitrogen (Van Slyke N). Ten "essential" amino acids have been quantitatively determined by microbiological methods in whole cabbage and in cathode fractions after electrodialysis. Arginine, threonine and lysine have been compared in whole cabbage, electrodialyzed, alcohol, and hot water extracts. The total nitrogen and Van Slyke N contents of these acids have been calculated and compared with the total N and Van Slyke N of whole cabbage and its separated fractions.

Total solids and total nitrogen values of the two strains of cabbage differed by as much as 30% and 16%, respectively, but the extractable solids and total nitrogen differed by only 2%.

Total solids and nitrogen for the combined dialysate (cathode and anode) and the 70% alcohol extract for each strain were in close agreement. The Van Slyke N of the Danish Copenhagen Market strain was about 6% higher in the dialysate and 9% higher in the alcohol extract than the Wisconsin strain. This was estimated to be due largely to the amide glutamine as determined by chemical methods described in a second paper of this series.

Hot water extracted a higher percentage of total N than either of the other methods. Total solids, total N and Van Slyke N in the various extracts and the residues from these extracts gave from 97 to 100% recovery based on the values found in the whole cabbage.

Distribution of total nitrogen in the cathode and anode fractions of the dialysate differed by as much as 10% in different runs; this was thought to be largely due to the destruction of the amide glutamine which was broken down by electrodialysis but not by alcohol extraction.

The amino acids arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine were recovered quantitatively in cathode and residue fractions after electrodialysis (±10%). Lysine recovery was somewhat variable in different sample runs and a little on the low side. Arginine recovery was about 20% high when the cathode was not hydrolyzed before analysis and the recovery from the Danish strain was a little higher than that from the Wisconsin strain. Tryptophan was partially destroyed by electrodialysis and yielded only 60% of that found in hydrolyzed whole cabbage.

Arginine was the only "essential" amino acid present in larger amounts in the soluble fraction that in the protein fraction.

Electrodialysis and alcohol extraction methods were equally suitable for separation of nine of the essential amino acids but alcohol extraction is preferred if other amino compounds are desired. Electrodialysis would be preferred as a method of separation of non-protein amino compounds from protein if the protein were to be used for further study.

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NITROGEN COMPOUNDS OF CABBAGE. II. CHROMATOGRAPHIC ANALYSIS OF THE NON-PROTEIN NITROGEN

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In the preceding study of this series *(4)*, the non-protein nitrogen fraction in its entirety was examined in relation to total nitrogen. Using microbiological procedures some ten "essential" amino acids of the non-protein and bulk protein fractions were assayed. However, it became clear that these ten amino acids represent only a small part of the non-protein nitrogen fraction; in fact, arginine might be described as the only "essential" amino acid in the non-protein fraction occurring in major quantities. The Kjeldahl nitrogen provided by these ten amino acids represents 22% of the entire nitrogen of the non-protein fraction.

Little has been published on the qualitative as well as quantitative make-up of the non-protein fraction of cabbage (cf Part I, this series) nor, with the exception of a few specific compounds, has it been examined in detail. Therefore, it was evident that a more complete examination was desirable in order to ascertain the components and quantitative distribution of a large segment of this fraction.

EXPERIMENTAL PROCEDURES

Materials and preparation of non-protein fraction. The Wisconsin and Danish strains of Copenhagen cabbage studied were of the same harvest and sampling as that used in Part I *(4)*. Segments of 19 heads of Wisconsin Copenhagen cabbage with a total fresh weight of 1750 g (129 g moisture-free basis) were ground in a Waring blender with a volume of 95% ethanol equal to 3 times the fresh weight of the cabbage. On filtration the ground cabbage residue was reextracted with portions of 70% ethanol until the washings gave only a weak ninhydrin-positive reaction. The residue was further extracted by soxhlet with hot 70% ethanol for 8 hr. All alcohol extracts were combined and concentrated on a rotary evaporator at 40° C under reduced pressure to a volume which represented 7.0 g fresh weight of cabbage/ml or 0.515 g moisture-free basis (MFB)/ml. The Danish strain was treated in like manner. The concentrate was made to a volume equal to 7.0 g fresh weight of cabbage/ml or 0.394 g MFB/ml.

Methods of analysis. The extract was analyzed for its amino- and imino-nitrogen constituents by a quantitative paper chromatographic procedure. It was not found necessary to desalt the extract prior to chromatography. The procedure employed was a modified version of the descending two-directional method of Steward and Thompson *(12)*. For convenience, sheets (18¼ x 22½ in) of prewashed Schleicher and Schuell No. 589 blue ribbon filter paper were employed in lieu of acid-washed Whatman No. 1. n-Butanol:glacial acetic acid:water (9:1:2.5) replaced the collidine-lutidine solvent *(12)* for the separation in the second direction. The compounds were located after development of the color with the ninhydrin reagent (1.0% in 95% ethanol) under the humidified, anaerobic conditions set forth by Steward, Zacharius and Thompson *(13)*. Colored spots were cut out of the paper chromatogram, cut into small rectangular pieces and eluted with 50% ethanol. The color densities were determined on a Beckman DU Spectrophotometer at 570 mμ for all compounds except pipecolic acid

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and proline; the latter was determined at 440 mmc. Interpretation of density values was made from standard curves prepared in advance for each of the compounds assayed; these standard values were checked at various time intervals.

Pipecolic acid was located on a two-directional paper chromatogram after reacting with 0.05% ninhydrin in 95% ethanol and heating the paper at 70° C for 10 min. The pipecolic acid spot was cut out, cut up into small squares, and reacted further with 4.0% ninhydrin in a test tube and the color density determined at 575 mmc according to the method of Silberstein *et al (11)*. No paper blank was found to be necessary. In agreement with Silberstein *et al* this method was not found applicable to the quantitative determination of proline.

The value obtained for histidine can only be considered approximate. Following a uni-directional separation of the extract with the n-butanol-acetic acid solvent, histidine was located from the orange-red color formed with freshly diazotized p-bromoaniline *(1)* sprayed on the paper chromatogram. The colored area was removed and its density determined with a Lumetron (Photovolt Corporation, New York, New York) after wrapping it around a test tube which fits the adapter. By revolving the tube a maximum reading could be obtained. A constant, K, was established using the formula

$$\frac{\text{mg of histidine}}{\text{maximum density x area}} = \text{K}.$$

The weight of the paper was taken as a measure of area. A mean value for K was determined in the range 2.5–15.0 mg of histidine and the amino acid values for the plant extracts were obtained using the K value.

Cystine was estimated as cysteic acid after oxidation with 30% hydrogen peroxide and 0.02% ammonium molybdate catalyst after application to the filter paper sheet.

Kjeldahl N values were obtained by the official A.O.A.C. *(7)* method using semi-micro Kjeldahl apparatus. Amino-nitrogen determinations were made by the Van Slyke method with a modified auxiliary reaction chamber as described by Doherty and Ogg *(3)*. Free ammonia was determined employing essentially the method of Pucher *et al (9)* modified by the inclusion of a trap between the distillation flask and connecting bulb consisting of an adapter filled with glass helices. The ammonia was collected in 4% boric acid and the titrations carried out with 0.02N HCl with Kjeldahl mixed indicator. Glutamine was determined both by the paper chromatography method described above and according to the ammonia method of Pucher *et al (9)*. Asparagine was also determined chromatographically by two different procedures and by ammonia difference according to Vickery and Meiss *(15)*. In an alternate chromatographic procedure for asparagine, the asparagine spot on the two-directional chromatogram was located with 0.05% ninhydrin, cut out and the determination, essentially followed that described by Connell *et al (2)*.

RESULTS AND DISCUSSION

Results of the analysis of the alcohol extractable nitrogen of Wisconsin Copenhagen cabbage are shown in Table 1.

A summation of amino-nitrogen furnished by the individual compounds assayed chromatographically accounts for 76% of the determined Van Slyke nitrogen value and the total nitrogen of these same compounds is equal to 60% of the Kjeldahl value. These figures appear reasonable in view of the fact that we have only assayed for the ninhydrin-positive nitrogen components of the fraction. The total nitrogen of the 10 "essential" amino acids represents 33% of the summed total nitrogen of the compounds determined. In terms of the summed Van Slyke nitrogen only 19% is contributed by these "essential" amino acids.

The major ninhydrin-positive component is glutamine while the amide of aspartic acid is present in less than one-fourth of that amount. It is noteworthy that S-methylcysteine sulfoxide represents both the major amino acid

TABLE 1

Chromatographic analysis of the non-protein nitrogen fraction of cabbage

Compound	Amino acid mg/g (MFB)	Expressed as V.S. amino N mg/g (MFB)	Expressed as total N mg/g (MFB)
Aspartic acid	2.2	0.22	0.22
Glutamic acid	3.4	0.32	0.32
Asparagine	2.2(2.0)[1]	0.23(0.21)[1]	0.45
Glutamine	10.0(10.68)[2]	1.7(1.84)[2]	1.9
Serine	2.2	0.3	0.3
Threonine	1.1	0.1	0.1
Alanine	3.0	0.47	0.47
Histidine	0.59	0.05	0.16
Lysine	0.43	0.08	0.08
Arginine	5.1	0.41	1.6
Methionine	0.25	0.02	0.02
Methionine sulfoxide	0.18	0.01	0.01
S-methylmethionine	0.03[3]	0.003	0.003
Cystine	0.16	0.02	0.02
S-methylcysteine sulfoxide	6.6	0.61	0.61
Valine	0.67	0.08	0.08
Leucine + isoleucine	1.1	0.11	0.11
Tyrosine	Trace	—	—
Phenylalanine	0.68	0.06	0.06
Tryptophan	0.0	0.0	0.0
γ-Aminobutyric acid	0.36	0.05	0.05
Proline	0.35	0.00	0.04
Pipecolic acid	0.54	0.00	0.06
Total		4.84	6.66

Van Slyke Amino N determination on extract ..... 6.34 mg/g (MFB)
Kjeldahl N ..... 11.1 mg/g (MFB)

[1] By a second ninhydrin method of color development.
[2] By Vickery's NH_3 method *(9)*.
[3] Calculated from Winteringham *(16)*.
V.S. = Van Slyke.

constituent as well as the sulfur-containing amino acid of this extract. The value reported here is in close agreement with that reported by Morris and Thompson *(8)* in a survey of *Brassicas*. The quantity of cysteine and methionine on a comparative basis is extremely small. Arginine is the third major amino compound present and because of its high nitrogen content is second only to glutamine on a total nitrogen basis. These three compounds make up 43% of the Van Slyke nitrogen of the fraction.

The glutamine value as obtained by quantitative paper chromatography was equal to 94% of that found by the ammonia method of Vickery (Table 2). This amide provided 29% of the Van Slyke nitrogen of the Wisconsin Copenhagen strain and 41% in the case of the Danish Copenhagen cabbage.

Efforts to determine asparagine by the ammonia method of Vickery had yielded very large and unreproducible values which were far in excess of the chromatographic ones. This is attributed to destruction by acid hydrolysis of S-methyl-cysteine sulfoxide with the concomitant production of ammonia *(8, 14)*. Thus, whenever the latter sulfur amino acid is present in the sample, ammonia production by even mild acid hydrolysis for the determination of asparagine (or other amides) must be excluded.

TABLE 2

Free ammonia and glutamine of the non-protein nitrogen fraction of cabbage

Sample	Free NH_3 mg/g (MFB)	Glutamine NH_3 mg/g (MFB)	Calculated glutamine (from NH_3) mg/g (MFB)	Calculated glutamine Van Slyke N (from NH_3) mg/g (MFB)	Van Slyke N mg/g (MFB)
Cabbage extract before buffer hydrolysis					
Strain 1[1]	0.706				6.34
Strain 2[2]	0.409				10.27
Cabbage extract after buffer hydrolysis					
Strain 1[1]		1.24	10.68	1.85	3.62
Strain 2[2]		2.84	24.70	4.22	4.78

[1] Strain 1 refers to Wisconsin Copenhagen cabbage.
[2] Strain 2 refers to Danish Copenhagen cabbage.

Although the Danish strain was found to contain a much higher level of Van Slyke nitrogen in the alcohol extractable fraction than the Wisconsin strain, 60% of this difference is immediately attributed to glutamine as calculated from columns 3 and 5 of Table 2.

It is interesting to note that the quantity of pipecolic acid present exceeds that of its proline analogue—a protein constituent—and is very similar in concentration to many of the "essential" amino acids in the non-protein fraction.

Quantities of pyrrolidone carboxylic acid were found present in the ethanol extract as well as evidence of some unidentified peptides and other simple ninhydrin-positive compounds. Conceivably the ring compound arose as an artifact from glutamine on extraction and storage of the extract. A large amount of an unidentified Erlich-reagent-positive compound, presumably an indole derivative, was also detected in the extract; the presence of tryptophan, however, could not be established.

S-methylmethionine could not be easily assayed by the above method because of its low concentration in the complex mixture wherein many of the components are present in considerably greater magnitude. The figure given is calculated from Winteringham *(16)*. It is rather remarkable that this sulfur amino acid was detected, isolated and identified *(6)* somewhat earlier than S-methylcysteine sulfoxide *(8, 14)* when one considers the concentration of each of these compounds.

Contrary to the findings of others *(5, 10)*, the presence of hydroxyproline could not be established in the non-protein nitrogen fraction. Furthermore, in agreement with the microbiological studies in Part I, and contrary to the published analysis of Majumder *et al (5)*, arginine represents one of the larger amino acid constituents of the non-protein nitrogen of cabbage.

Any differences between the values reported here and those for the 10 amino acids in Part I (cf Table 3) may be attributed to both differences in preparation of the non-protein nitrogen fraction and methods of assay. Microbiological assay values obtained on the unhydrolyzed cathode appeared to reflect unknown growth factors—possibly attributable to peptides. Chromatographic determinations reported in this paper were made on an unhydrolyzed aqueous ethanol extract. Some of the differences between the latter

values and the hydrolyzed cathode presumably resulted from peptide hydrolysis. Moreover, it was shown *(4)* that the extraction of free lysine by aqueous ethanol was incomplete when compared with the cathode or hot water extracts.

SUMMARY

Results of the chromatographic analysis of the non-protein nitrogen of Wisconsin Copenhagen cabbage are given.

Glutamine represents the major amino-nitrogen compound of the non-protein nitrogen fraction of cabbage. S-methylcysteine sulfoxide is second in concentration in this fraction while arginine represents a third major amino constituent. These three compounds provide 43% of the Van Slyke nitrogen of this fraction. The individual components assayed by the chromatographic method account for 76% of the Van Slyke nitrogen value and 60% of the Kjeldahl value.

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THE ISOLATION AND PARTIAL CHARACTERIZATION OF A VOLATILE ESSENTIAL OIL FRACTION FROM BITTER CANNED CARROTS [a, b]

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(Manuscript received May 21, 1959)

The problem of bitter flavor in carrots, particularly from cold storage *(2, 3, 14)* has received considerable attention. The bitter flavor of carrots may be empirically divided into two parts *(10, 14)*. One part is nonvolatile, and Sondheimer *(9, 11)* and Dodson, Fuki, Carolus and Sell *(6)* have isolated a bitter nonvolatile compound (Compound A) from carrots later identified by Sondheimer *(10, 11)* as 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin. This compound has an ultraviolet absorption spectrum similar to hexane extracts of bitter carrots *(9)*, and appears to be largely responsible for the nonvolatile portion of bitter carrot flavor.

The second empirical part of bitter carrot flavor is volatile and steam distillable *(10, 14)*, and also has a characteristic ultraviolet absorption spectrum *(10)*. In continuation of the chemical studies of Sondheimer, we have focused our attention on the volatile phase of bitter carrot flavor with the hope that the additional information would suggest the aberrant physiological or biochemical mechanism for bitter carrot flavor. Descriptions of the components of carrot root flavor could not be found in the literature. This is understandable since the volatile components of carrot root flavor are in exceedingly low concentration. This report describes the isolation and partial characterization of the volatile flavor of bitter canned carrots.

EXPERIMENTAL

Canned bitter carrots *(9)* weighing 122 kg fresh basis were ground in a one-gallon capacity Waring blender and steam distilled in the pilot plant. The distillate, obtained by cooling the vapor to 15° C, and judged complete by the absence of odor in the residue, was concentrated to a volume of 4 liters by repeated steam distillation.

The volatile flavor was further concentrated by extracting the distillate twice with one quarter volumes of ethyl ether, which was then dried over anhydrous sodium sulfate. The ether and residual water were completely removed by evaporation *in vacuo* and filtering through kieselgur. The resulting oil was stored at −10° C prior to analysis.

Specific gravity measurements were obtained by use of a decimeter pycnometer, and refractive indices were obtained using an Abbe refractometer. Melting points were determined on a microscope hot stage, and ultraviolet measurements were made in a Beckman Model DU spectrophotometer. Flavor panel evaluations were made with a 10–12 membered panel employing the triangle-intensity test of Davis and Hanson *(5)*.

RESULTS AND DISCUSSION

Steam distillates of canned bitter carrots are bitter and are further characterized by the presence of a milk-like emulsion. After distilling 122 kg bitter

[a] Journal Paper No. 1182, New York State Agricultural Experiment Station, Cornell University, Geneva, New York.

[b] No. V. in the series "Bitter Flavor in Carrots." For I–IV see references 2 and 9–11.

carrots and extracting with ether, 3.1 g highly refractive, $n^{22/D} = 1.5150$, and dense, $d^{20/20} = 1.061$, pale yellow oil were obtained. The oil had a bitter, harsh, hot flavor and completely accounted for the volatile bitter flavor of carrots as can be seen from the organoleptic evaluation of the fractionation

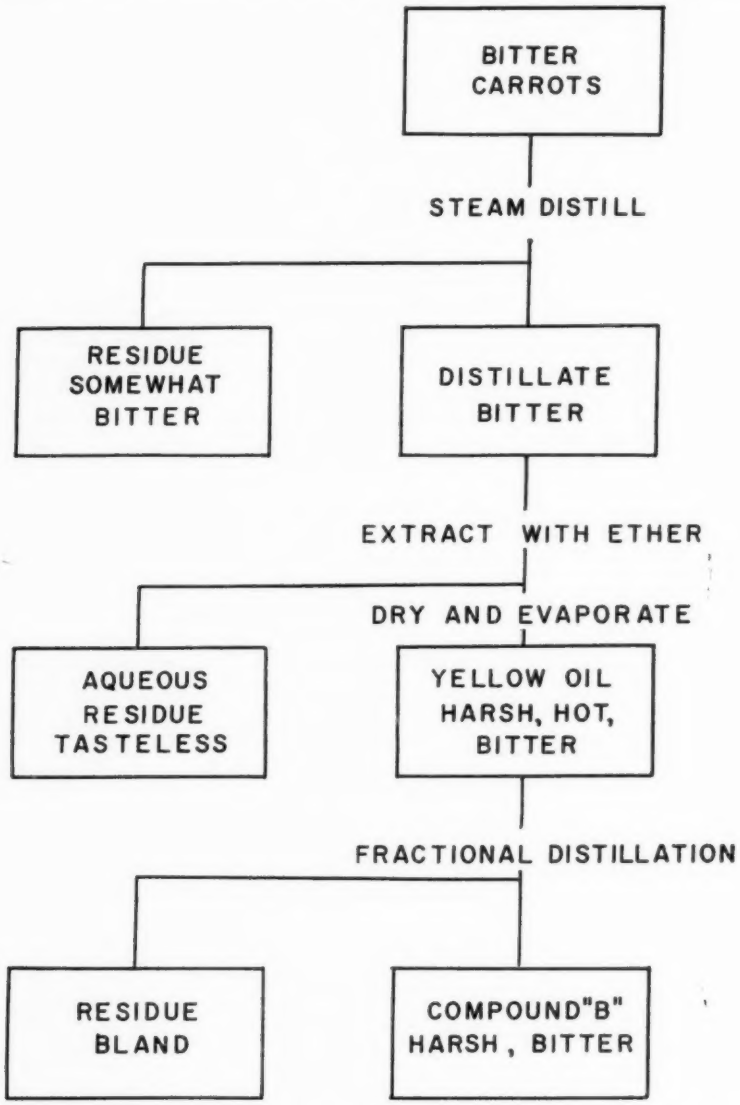

Figure 1. Organoleptic evaluation of the fractionation scheme used for the volatile components of canned bitter carrot flavor.

scheme shown in Figure 1. The oil also had an ultraviolet absorption spectrum (Figure 2) strikingly similar to that reported by Sondheimer *(10)* for steam distillates of bitter carrots.

It was concluded that the total volatile oil of canned bitter carrots was a sesquiterpene or mixture of sesquiterpenes on the basis of the following observations:

The total volatile oil was neutral toward litmus, soluble in acetone, ether, petroleum ether, H_2SO_4, and about 20 volumes 95% ethyl alcohol. The total oil was insoluble in H_2O, 5% NaOH, H_3PO_4, bisulfite solution and HCl. It discolored 4% $KMnO_4$. Fusion with metallic sodium established the absence of sulfur, nitrogen, and halogen. A boiling point of 136-138° C. at 3 mm. Hg was recorded using micro methods but was not considered accurate due to a tendency of the oil to resinify during the determination. Distillation in an Emich tube *(4)* over a micro flame resulted in decomposition of the total oil with the production of a dense black smoke and generation of phenols (blue color with $FeCl_3$ solution).

Fractionation of the total oil. Fractional distillation of the oil was attempted in a semimicro distillation apparatus at 3 mm. pressure. One and one-tenth grams pale yellow oil boiling at 108° C were obtained from 2.8 g total oil. Prolonged distillation, bath temperature 245° C, failed to produce additional fractions. The distillable fraction was harsh and bitter, whereas the residue, which showed a tendency to resinify and crystallize (mp 90-110),

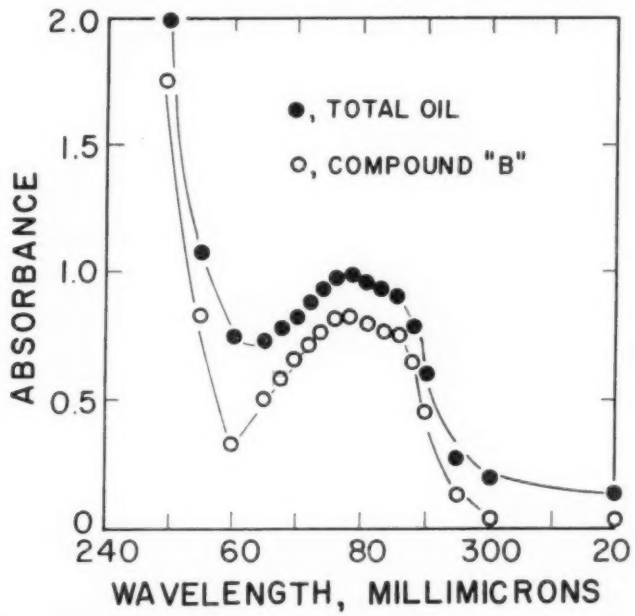

Figure 2. Absorption spectrum of essential oil of canned bitter carrots and of the major flavor component, compound B. C = 200 mg per liter.

was essentially tasteless (Figure 1). It was concluded that the distillable fraction, referred to as compound B, was similar to the carotol or daucol reported by others *(1, 7, 8, 12, 13)* to be components of carrot seed oil on the basis of the following measurements: bp at 3 mm pressure, 108° C, $n^{22/D} = 1.5074$, $d^{20/20} = 0.9628$, $[\alpha]\ 20^{/D} = +5.14$. Anal. calculated for $C_{14}H_{18}O_2$, C = 77.06%, H = 8.26%, O = 14.67%, M.W. = 218. Found, C = 76.52%, H = 8.34%, O = 15.27%, M.W. (Rast) = 217.4.

Compound B gave inconclusive results for functional group tests (alcohols, aldehydes, and esters). It (compound B) is to be distinguished from carotol or 1-7-dimethyl-4-isopropyl naphthalene in that compound B possesses the distinct flavor of the carrot root and also has characteristic ultraviolet absorbance (Figure 2). Carotol shows no ultraviolet absorbance, and possesses, moreover, the flavor of the carrot seed. While compound B possesses two molecules of oxygen it is to be distinguished from daucol *(1, 8, 12, 13)* in that the latter is a solid melting at 115–116° C.

Finally, while compound B corresponds well with the empirical formula $C_{14}H_{18}O_2$, it cannot be concluded at this time that the material is not a 15 carbon sesquiterpene. Due to entrainment during fractional distillation there is good possibility that compound B is as yet too impure for accurate analysis.

Flavor tests. Compound B exists in bitter carrots at a concentration of at least 0.001% (by isolation). A flavor panel *(5)* can significantly detect this material when added to carrots at a concentration of 0.001%. Since isolation schemes are seldom quantitative compound B probably exists in higher concentration in canned bitter carrots. The opinion of several panel members and also persons representing interested carrot processing companies is that the material called compound B is typical of carrot flavor and characterizes well, the bitter flavor problem in carrots.

SUMMARY

A volatile essential oil, presumably a sesquiterpene or mixture of sesquiterpenes has been isolated from canned bitter carrots. It is suggested that the volatile bitter oil is a component of bitter flavor. The oil is partially characterized.

Acknowledgment

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THE CONSTITUENTS OF THE CRYSTALLINE DEPOSITS ON DRIED FRUIT[a]

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"Sugaring" is a type of deterioration which occurs on dried fruits and commonly appears under packing house conditions. "Sugar" deposits occur more commonly on the stored unprocessed fruit than on packaged fruit. The condition occurs very frequently in dried prunes and figs and less commonly in other dried fruits.

The sugary substance appears as a deposition either on the surface or immediately under the skin of the dried fruit. It is powdery to granular in texture and white to brownish in color. When the deposit occurs under the skin of prunes, it is called "red sugar." The material is soluble in water but not easily removed from the fruit.

While the conditions which cause the formation of these substances are not known the deposition can be minimized by raising the moisture content of dried fruit to approximately 24% moisture, and by "sweating"[b] the processed fruits under controlled conditions.

The appearance of "sugar" on natural-condition dried fruits and in processed fruits is unsightly and has led to the belief that microbial spoilage is associated with the condition. While "sugaring" has probably existed as long as fruits have been dried, there are but few studies concerning this condition. An investigation of this problem was done by Mrak and Baker (1939), when they studied the sugar-tolerant yeasts responsible for the spoilage of dried fruits. Their examination of the material found on figs and prunes obtained from storage bins at processing plants showed that, in addition to sugar, appreciable numbers of yeasts were present. They found that the deposits consisted of glucose and, occasionally, very small quantities of sucrose. Fructose was not found in any of the samples analyzed. The moisture content was generally between 8 and 10 per cent. Twenty-five to 44% of the prune "sugar" and 6 to 21% of the material from figs was composed principally of yeast cells. The number of yeast cells found in "sugar" from fruit out of the bin was much higher than that found in the deposits from any of the packaged samples. Some packaged samples had no yeast cells at all. These authors concluded that it would be possible to control this type of deterioration by dehydration or rapid sun-drying to a relatively low moisture content, fumigation with ethylene oxide and proper storage.

Since 1938, there have been considerable changes made in the production and storage of dried fruits. An example of this is in prunes. In 1938, a considerable number of prunes were still sun-dried whereas today virtually all

[a] Presented at the Nineteenth Annual Meeting of the Institute of Food Technologists, May 18, 1959, Philadelphia, Pennsylvania.

[b] "Sweating"—A curing operation, storage at an elevated temperature to equilibrate the processed fruit.

prunes are dehydrated. Storage of the unprocessed prunes at that time was in large bins, each holding up to 40 tons of dried fruit. Today, the fruit is generally held in 1 ton palletized bins which allow more air movement and reduce the possibility of incipient fermentation.

Many figs, while still sun-dried, now are finished in dehydrators at a relatively low temperature. The dried figs are then stored in large shallow "sweat" boxes.

Attempts to control the storage atmospheres and temperatures are much more prevalent today than they were in the 1930's. Controlled humidities and low temperatures are used by many packers for the storage of dried fruits.

The purpose of this study was two fold. First, a complete analysis of the deposits using modern methods was made and second a survey was made of the microorganisms associated with the "sugaring" condition under the present conditions of production, storage and processing.

MATERIALS AND METHODS

Samples of five different types of dried fruit were collected from commercial sources. They included sun-dried, dehydrated and processed fruits. Figs were of the Calimyrna, Adriatic, Mission and Kadota varieties, and included sun-dried and sun dried-dehydrated samples. The two varieties of prunes investigated, French and Robe, were dehydrated. Thompson seedless raisins, Blenheim apricots, and Elberta peaches, all of which were sun-dried, were also studied.

Samples of the "sugary" deposition were collected by two means. The first consisted of brushing the surface of the dried fruit with a stiff-bristled brush and collecting the

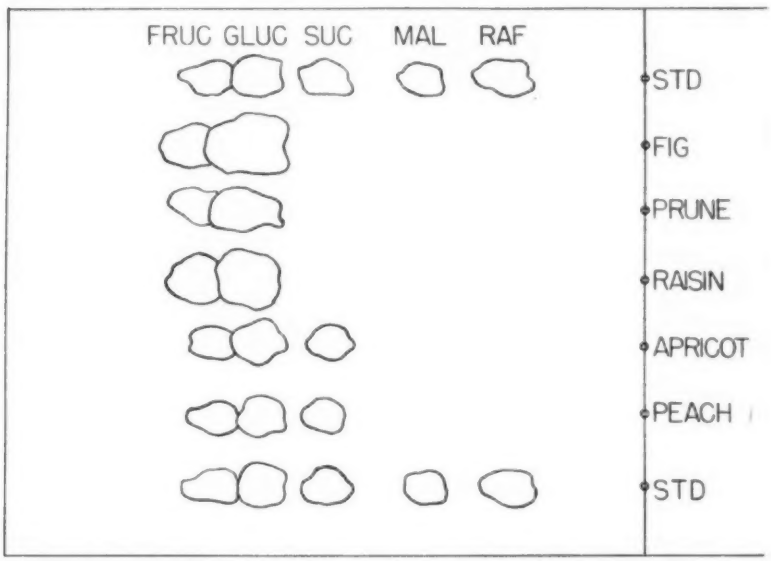

Figure 1. Chromatogram showing the sugars present in the "sugary" deposits on dried fruits. FRUC = Fructose, GLUC = Glucose, SUC = Sucrose, MAL = Maltose, RAF = Raffinose.

deposit which was removed by this action. The second was to remove the skin of the dried fruit over the sub-epidermal depositions by a scalpel and removing the "sugary" material. The "sugars" collected by these two methods were analyzed separately.

Examination for the presence of microorganisms in the sugar deposit was made by plating and microscopic examination. Aliquots of the various "sugars" were plated on three different media: Bacto-eugon agar (Difco), 10% malt extract agar, and a high sugar medium consisting of 35% cerulose, 10% malt extract, and agar. Counts were made for bacteria, yeast and molds, and for osmophilic microorganisms. In addition, the "sugars" were suspended in water and examined under a compound microscope to determine whether or not yeast cells were present.

Determination of the chemical composition of the "sugars" was made by chromatographic techniques. Initially, bidirectional chromatograms were prepared, but due to the limited number of compounds found, unidirectional chromatograms were used for the majority of the study. The chromatograms were prepared by using a descending solvent front on Whatman #1 paper. Solvents used for the bidimensional chromatograms were as follows: For amino acids and sugars the first solvent was phenol: water (80:20); the second solvent was butanol: acetic acid: water (5:1:4). For organic acids the first solvent was ethanol: ammonium hydroxide: water (95:6:18); the second solvent was butanol: acetic acid: water (5:1:4). For the unidirectional chromatograms the following solvents were used: For sugars isopropanol: ethanol: water (7:1:2). For the organic and amino acids the following solvent was used: n-butanol: formic acid: water (4:1:5). Sprays used to develop the spots were as follows: For sugars, silver nitrate in acetone and benzidine TCA, organic acids, brom phenol blue in ethanol. Amino acid spots were developed with ninhydrin in acetone (developed in 70° oven 15 minutes). (Block *et al.* 1955).

The "sugar" was dissolved in water to give a concentration of 100 mg per ml from which 0.005 ml was used for chromatographic spot. Standards of known compounds were run simultaneously in the unidirectional chromatograms.

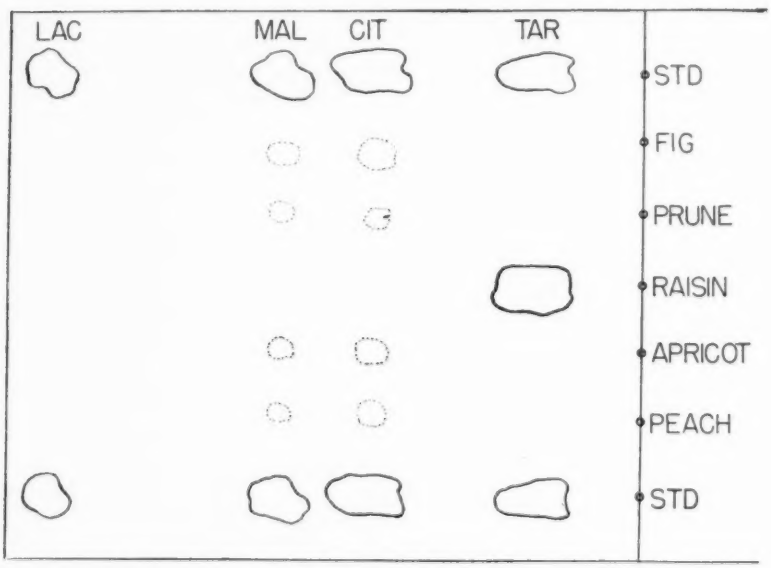

Figure 2. Chromatogram showing the organic acids which occur in the "sugary" deposits on dried fruits. LAC = Lactic Acid, MAL = Malic Acid, CIT = Citric Acid, TAR = Tartaric Acid.

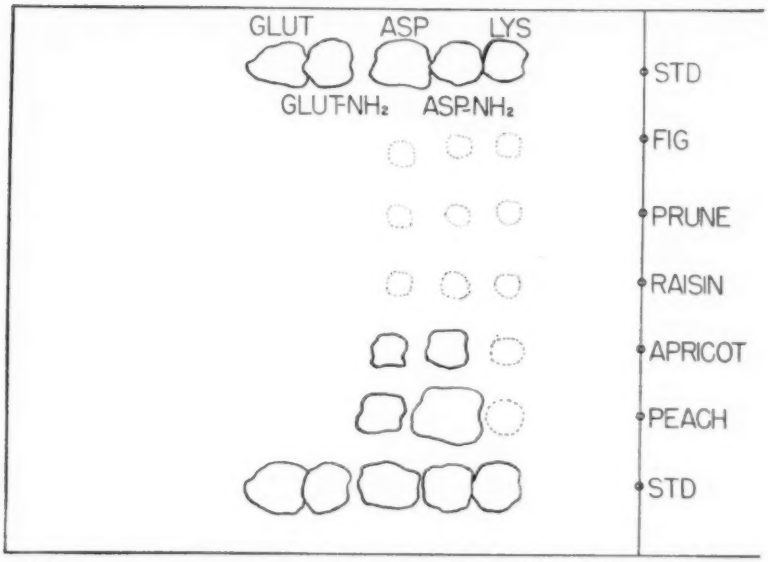

Figure 3. Chromatogram showing the amino acids present in the "sugary" deposits on dried fruit. GLUT = Glutamic Acid, GLUT-NH_2 = Glutamine, ASP = Aspartic Acid, ASP-NH_2 = Asparagine, LYS = Lysine.

EXPERIMENTAL RESULTS

The chromatographic separation of the constituents of the sugary deposits showed that they were composed of very few compounds. All of the samples analyzed contained glucose and fructose as major constituents. The "sugar" obtained from apricot, peach and prune samples contained more glucose than fructose. Fig and raisin "sugars," on the other hand, had approximately equal proportions of glucose and fructose. Sucrose

TABLE 1

Fruit variety	No. of samples	Sugars	Organic acids	Amino acids
Apricots Blenheim	5	larger amount of glucose, lesser amount of fructose, small amount of sucrose	traces of citric and malic acids	large amount of asparagine, small amount of aspartic acid, trace of lysine
Figs Adriatic Calimyrna Kadota Mission	14	*ca.* equal amounts of glucose & fructose	traces of citric and malic acids	traces of lysine, asparagine and aspartic acid
Peaches Elberta	2	similar to apricots	similar to apricots	similar to apricots
Prunes French Robe	10	large amount of glucose, lesser amount of fructose	similar to figs	similar to figs
Raisins Thompson Seedless	2	similar to figs	large amount of tartaric acid	similar to figs

was observed only in the samples obtained from apricots and peaches and even then in fairly small amounts. (See Fig. 1).

There was no appreciable quantity of organic acids found in any of the samples with the exception of raisins, which contained a large amount of tartaric acid. The other samples contained trace amounts of citric and malic acids. (See Fig. 2).

The amino acids lysine, asparagine and aspartic acid were also found in trace amounts with the exceptions of apricots and peaches. Samples of "sugars" from these cut fruits contained large amounts of asparagine and small amounts of aspartic acid. (See Fig. 3).

Table I summarizes the chromatographic data collected on the "sugar" depositions analyzed in this study.

There were no detectable amounts of any other sugars, organic acids or amino acids found in the preparations. The "sugar" collected from different varieties of figs, and of prunes, showed no differences nor were differences demonstrated between surface and sub-epidermal depositions.

The results, in general, of plating aliquots of the various "sugar" samples on the three different media were negative. In a few cases, bacterial colonies were found on the plates, one or two mold colonies, and a yeast-like mold identified as *Sporobolomyces*. The latter organism is commonly found on leaves and in organic humus and is incapable of growth on a concentrated sugar medium. All of these may be considered as incidental contaminations of the samples.

Microscopic examinations confirmed the negative results obtained by plating the samples.

The lack of significant numbers of any microorganisms and total absence of any sugar tolerant organisms in all of the samples analyzed would indicate that, under the present day conditions of preparing and handling dried fruits, "sugaring" is a separate and distinct type of deterioration from that of microbial spoilage.

SUMMARY

The constituents of the crystalline deposits, responsible for the type of deterioration known as "sugaring", on dried fruits have been determined by the use of paper chromatographic techniques.

Deposits found on prunes and figs contained glucose and fructose, traces of citric and malic acids and of lysine, asparagine, and aspartic acid. Apricot and peach "sugars" contained, in addition, some sucrose and large amounts of asparagine and aspartic acid. Deposits from raisins were similar to figs with the exception that they also contained large amounts of tartaric acid.

Plating and microscopic examinations did not reveal the presence of yeasts which had been previously reported as a constituent of the "sugar" depositions. This difference from the results of an investigation of the "sugaring" problem 20 years ago might be the consequence of the different handling and drying techniques used now.

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THE RED PIGMENT OF THE ROOT OF THE BEET (BETA VULGARIS) AS A PYRROLE COMPOUND[a]

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The red pigment in the root tissue of the red varieties of beets, betanin, investigated during the past 40 years, has been assumed to be a "nitrogenous anthocyanin." That this pigment actually contains an anthocyanin nucleus attached in some way to a nitrogenous compound has not, however, been shown unequivocally. Previously published data and new observations on the spectral properties (absorption spectra in ultra violet and infra-red regions) and reactions of chromatographically pure preparations have led us to postulate that the nitrogenous red pigment is a pyrrole derivative. This was reported briefly previously *(7)*. The data obtained are presented in more detail in this article.

Literature review. Investigators in the field have been plagued by the instability of betanin. Unlike typical anthocyanins, the beet pigment cannot be hydrolyzed to its aglucone by acid hydrolysis without complete decomposition *(16)*. Most of the early investigations appeared to indicate that it was similar to the known anthocyanins even though no previous "nitrogenous anthocyanin" had been discovered. Robinson and co-workers *(1, 10, 11)*, who synthesized polyhydroxy flavylium types which contained nitrogen in various positions around the flavylium nucleus, showed that betanin is not an N-acylated amino anthocyanin *(10)*, but found definite similarities to the 4′ amino flavylium salts *(11)*. These possess a color, stability to cold mineral acids, and behaviour to alkali quite similar to the respective properties of betanin. Betanin, however, was less stable in hot mineral acids and, oddly, more resistant to the action of nitrous acid than were the synthetic salts. This last property would now appear to be of utmost importance, although it was more or less forgotten by later workers. Recently, we reported *(7)* that betanin cannot be an anthocyanin and indeed is not even an aromatic compound. However, the question remains open *(18, 19)*.

Ainley and Robinson *(1)* showed that N-methyl, C-methyl and methoxyl groups were not present in the pigment molecule. No degradation products were obtained, although they reported the evolution of a base with methylamine like odor upon alkali fusion and a degradation fraction which gave a positive Ehrlich's test *(1)*. The pigment itself became methylated very easily; when hydrogen chloride was passed through a methanol solution of betanidin, 2 methyl groups were introduced. It was believed that one of the esterified groups was a carboxyl and the other probably a phenolic hydroxyl; the suggested formula $C_{20}H_{19\text{-}23}O_7N_2Cl\text{–}3H_2O$ would allow only one carboxyl group since two carboxyls would leave only 2 hydroxyls present, and these

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would not suffice to give the amino hydroxy flavylium compound the deep color characteristic of betanidin. Other evidence *(14)*, especially some recent electrophoretic data *(6, 17)* indicated that 2 carboxyl groups is a more reasonable assumption—which is additional evidence against the "nitrogenous anthocyanin" theory.

Evidence was presented in favor of the possibility that one of the 2 nitrogens in betanin is amino, but this was later disproved *(8, 13)*. Indeed, the behavior of the pigment with nitrous acid is quite unique. In the cold, there is no reaction; under usual Van Slyke conditions, 18% of the nitrogen present is released *(13)*; and upon warming, the whole pigment molecule is destroyed.

Betanin was finally crystallized in 1957 by Wyler and Dreiding *(17)*. Analysis of the crystalline preparation showed the absence of ash, chloride, N-methyl, C-methyl and methoxyl groups. Its nitrogen content was close to 6%. The most important single observation reported however, was the virtual absence of ultraviolet absorption although this was not mentioned at the time. This evidence would seem to rule out the occurrence of an aromatic nucleus in betanin. In a paper appearing only shortly after that of Wyler and Dreiding, Schmidt and Schönleben *(15)* reported the crystallization of the potassium salt of betanin. Their preparation contained about 5.5% potassium and 4% nitrogen.

Crude betanin preparations have been purified by column chromatography *(2)*. It is easily shown that not one, but many different, yet similar pigments occur in the common beet. Some do not contain nitrogen and it appears that under certain conditions, nitrogen-free yellow pigments may be precursors of red nitrogen-free pigments *(7)*, although the major red pigment is certainly nitrogenous.

EXPERIMENTAL

The beets used in these investigations were obtained from the Department of Vegetable Crops at Davis. The 5 varieties used were Detroit Dark Red, Extra Early Flat Egyptian, Green Top Bunching, Crosby's Egyptian (originally a selection from Green Top Bunching) and Early Wonder (originally a selection from Crosby's Egyptian). All the beets were grown during the 1956 or 1957 season, harvested in early December, topped, washed carefully and stored in cloth bags at 32° F for 3 to 6 months. When the roots had lost much of their moisture and were quite shrivelled, they were ground and dried on trays in a vacuum oven at 70° C for 4 hr. These dry preparations were stored in closed, dark jars at room temperature until used.

In general, purification of betanin was carried out by the methods of Schmidt and Schönleben, *(14, 15)* Wyler and Dreiding *(17)*, and Pucher, Curtis and Vickery *(8)*. The initial water or acid alcohol extract from the crude, dried beet material, however, was first fractionated on a magnesol-hyflo 1:1 column to isolate individual pigments. Ultraviolet absorption between 235 and 300 mμ was used as a check of purity during isolation. A modification of column electrophoresis was used for final purification of some of the pigments. The apparatus was based on the design suggested by Professor J. B. Neilands and used in large scale protein (enzyme) purification. The column was horizontal, 1 in square by 1 ft long. A filter paper wick extended from each end of the column into a buffer reservoir containing the electrode. The column was packed with 1-in cubes of very fine sponge synthetic rubber, previously wetted with buffer. After separation, the pigment was eluted merely by removing the small cube of sponge rubber containing the pigment zone and squeezing it out into a small container for eventual crystallization.

Hydrogenations were carried out in glacial acetic acid at 25° C using freshly prepared platinum oxide as catalyst *(4)*. Reduction was accomplished with zinc dust in acid solution, or with sodium borohydride or hydrazine sulfate in neutral solution.

Nitrogen was determined by the micro Kjeldahl procedure.

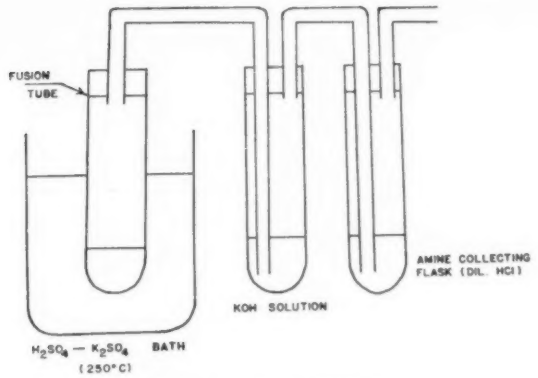


Figure 1. Fusion apparatus.

Fusions were carried out in the simplified apparatus shown in Figure 1. The volatile breakdown products obtained were thus collected quantitatively. The solid residue in the fusion tube was then acidified to drive out the remaining CO_2 into the CO_2 collecting flask. Methyl amine was identified using paper chromatography and determined colorimetrically by reaction with 1,2-naphthoquinone 4 sulfonate *(3, 12)*. The acidified residue was then extracted with 95% ethanol to remove the remaining breakdown products. The pyrrole derivative was purified by mercury precipitation from alcohol followed by decomposition of the mercuric salt with H_2S. Presence of the pyrrole compound was followed by spot tests using an Ehrlich's reagent containing 5% p-dimethylaminobenzaldehyde in concentrated HCl.

Infra red spectra were obtained using Beckman IR-2 and Perkin-Elmer Infrared spectrophotometers. Pigment samples were prepared in KBr pellet form by the usual procedures. Ultraviolet and visible spectra were obtained using a Cary model 11 recording spectrophotometer.

RESULTS AND DISCUSSION

Absorption spectrum. The absorption spectrum of crystalline betanin, Figure 2, is similar to that first reported by Wyler and Dreiding. The virtual absence of absorption in the near ultraviolet shows the absence of aromaticity. This is confirmed by the infra red data shown in Figure 3. There is no aromatic C-H stretching peak at 3.3 mc. Neither are there typical aromatic peaks near 6.2 and 6.7 mc. We are therefore not dealing with a "nitrogenous anthocyanin"—or any other aromatic molecule.

When one considers the probability of the nitrogen being in a ring *(8)*, and further, that it is only very weakly basic (or as now seems likely, slightly acidic) only one conclusion is tenable. The only known ring-nitrogen system with little or no aromatic character in which the nitrogen is not basic is pyrrole. Every known physical and chemical property of betanin supports this conclusion *(7)*.

Alkali fusion. Fusion of betanin with KOH was carried out in the apparatus described. The temperature was held at 250° C ($H_2SO_4 - K_2SO_4$ bath) for 10 min or longer to drive over the last traces of volatile amine. This was collected in hydrochloric acid solution, evaporated, and found to be a primary amine by the Hinsberg nitrous acid test. The solution containing

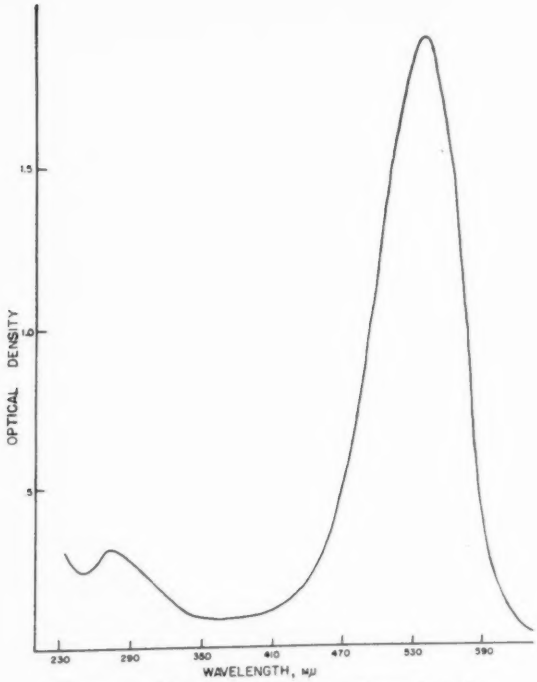


Figure 2. Absorption spectrum of pure betanin.

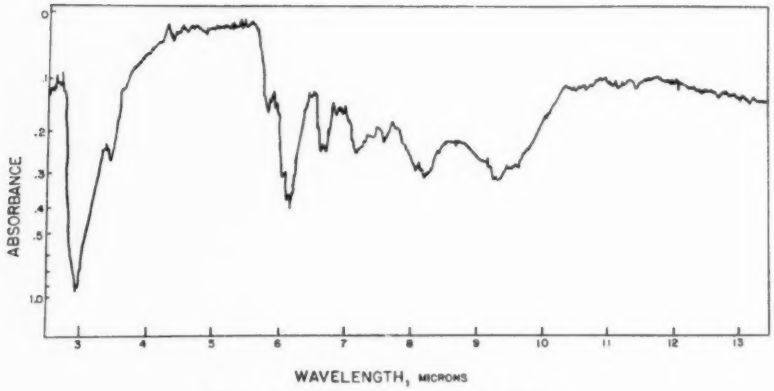


Figure 3. Infra red spectrum of betanin.

the amine hydrochloride after evaporation to dryness, was spotted onto Whatman no 1 filter paper strips for descending paper chromatographic development using butanol-acetic acid-water (4:1:5) and m-cresol-acetic acid-water (50:2:48). Ninhydrin spray was used to detect the spots. As shown in Table 1, only one compound, methyl amine, was present.

TABLE 1

Rf values

	BuOH-HAc-H_2O	m-Cresol-HAc-H_2O
Methyl amine	.37	.52
Ethyl amine	.45	.67
I-Propyl amine	.57	.75
Unknown (only one spot)	.37	.52

Presence of carbon dioxide as a breakdown product was shown by acidifying the residue in the fusion tube. The gas evolved was passed into a solution of lime water yielding a white precipitate.

The pyrrole derivative formed in the fusion was precipitated from the alcohol solution by the dropwise addition of 4% mercuric acetate solution. Decomposition of this precipitate in a water suspension with hydrogen sulfide gas bubbled through, and filtering gave a clear, almost colorless solution which was strongly Erhlich positive. When concentrated in a vacuum desiccator for a few hours, the material became thick and was no longer Ehrlich positive. No derivative was obtained and identified from this. It is well known that many pyrrole compounds polymerize under such conditions. Further attempts to crystallize the derivative after methylation may be fruitful.

Oxidation and reduction. Betanin is rapidly destroyed by most oxidizing agents and can be very accurately titrated with potassium permanganate to a colorless or slightly brownish end point *(9)*. Chromatographically pure water solutions of betanin which had just been removed from a magnesol column were accurately determined by this procedure. The titrated solutions were acidified with sulfuric acid, evaporated to low volume, and transferred quantitatively to micro Kjeldahl flasks for N determination. In this way, small amounts of pigment in solution were analyzed for nitrogen without first obtaining a solid sample.

A curious and extremely interesting feature of the chemistry of betanin is its reaction with nitrous acid. At 0° C, there is no reaction; yet at room temperature, the red color is rapidly and entirely destroyed. Pucher *et al (9)* found that about 18% of the nitrogen present was liberated under the usual Van Slyke conditions. They pointed out also that phenolic substances that are non-nitrogenous yield gas in the common Van Slyke determination. Reaction products from the nitrous acid degradation have not been identified.

Betanin was found to be easily reduced by zinc dust in acid solution to a yellow compound. Upon standing at room temperature, however, the yellow pigment again changed to a bright, clear red. The red pigment could again be reduced to yellow and reoxidized to red, etc. The absorption spectra of these 3 preparations are shown in Figure 4. It can be seen that the reduction was not precisely reversible; the final red pigment was not identical with the original purple betanin.

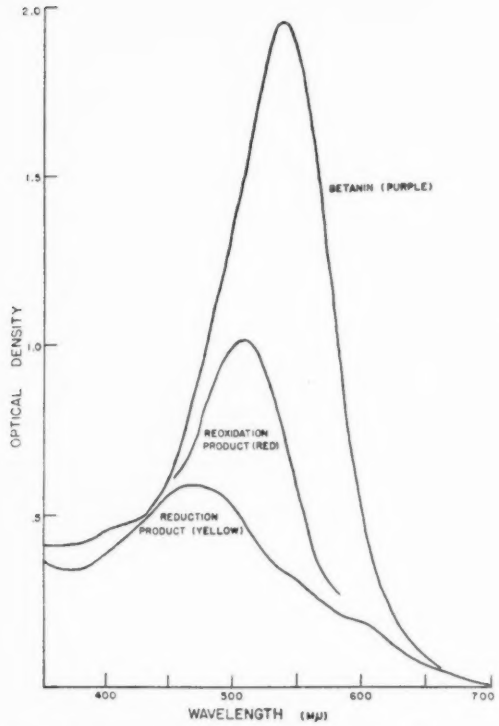


Figure 4. Reduction and reoxidation products of betanin.

A drop of the yellow (reduced) solution was spotted onto a piece of filter paper and allowed to dry. After about 1 hr at room temperature the spot was again a purple-red color, although not as intense as the original, unreduced purple. The yellow spot gave the following reactions in 10 min at 20° C with several spray reagents:

Spray Reagent	Color
Vanillin–HCl	Bright yellow, stable
H_2O_2	Pink-purple
Benzidine–HCl	No reaction

Betanin was also reduced to the yellow compound by sodium borohydride, but hydrogenation at 25° C in acetic acid solution using a freshly prepared PtO catalyst failed to occur.

During isolation and purification of the pigment, when the solution was held at room temperature in the presence of strong acid for several hours, the red color was destroyed and a brown precipitate formed. In some cases no precipitate formed, but the solution turned brown. When the brown solution was passed over a magnesol column, 2 zones formed. The first, a yellow zone,

moved down the column slowly; the second, brown, remained at the top of the magnesol. When either the brown precipitate or the brown magnesol (removed from the column top) was placed in water suspension and reduced with sodium borohydride or zinc in acid, the color changed through red to

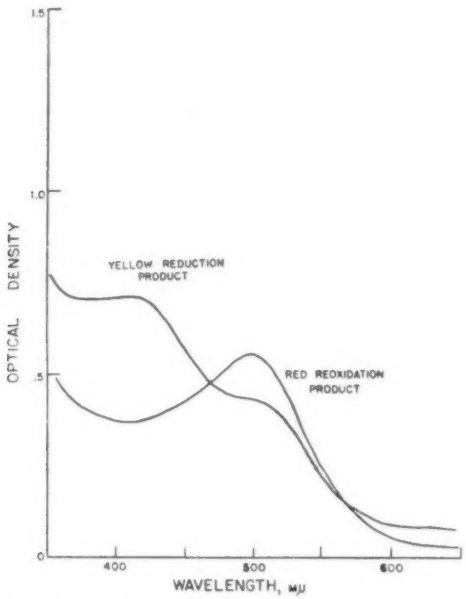
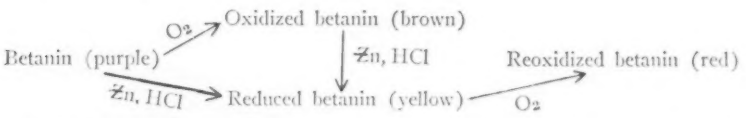


Figure 5. Spectra of the yellow and the red reduction products.

yellow. This yellow solution, allowed to reoxidize, returned to a clear, bright red solution. These spectra are shown in Figure 5. The changes taking place during these experiments may be represented thus:



Aromatic amine coupling. In working with the yellow pigments, in some cases, a chromatographically pure pigment eluted from the yellow band, changed color to orange in water solution on overnight storage at room temperature. The orange solution then was easily separated into a slow moving red pigment and a faster moving yellow on a fresh magnesol column identical with the one from which the first yellow band had been isolated. Thus, in some cases, it appears that some of the yellow pigments may be immediate precursors of the red ones.

It was also found that at least one yellow pigment would react with ortho or para substituted aromatic amines in acid solution at room temperature, to

give red to purple compounds in only a very few minutes. Not all of the yellow pigments present in the beet root are reactive. The yellow reduction product of the red betanin, for example, does not react, nor do some of the minor yellow fractions. In general, the major yellow pigment which moves just ahead of the main red pigment on the magnesol column reacts. It does not lose this reactivity readily, since it can be further purified first by precipitation as the lead or mercuric salt and regenerated in acid alcohol solution after isolation on either a magnesol or an anion exchange column.

The pink compound formed by the reaction between the yellow pigment and benzidine in acid solution was extracted by butanol from the acid solution. Addition of excess ether caused a pink gelatinous precipitate to form, but free benzidine was shown to be present and a further effort to purify the compound was not made. The purple solution resulting from the reaction between the yellow pigment and p-anisidine was passed over a magnesol column. It was shown that the original yellow compound was completely used up leaving a thin, fast moving brown band followed by the blue-violet product. The same yellow pigment was reduced slowly by zinc in dilute hydrochloric acid to a colorless solution. This colorless solution then no longer reacted with aromatic amines.

Nitrogen-free pigments. The major yellow pigment was separated as an extremely hygroscopic amorphous material by the addition of acetone to

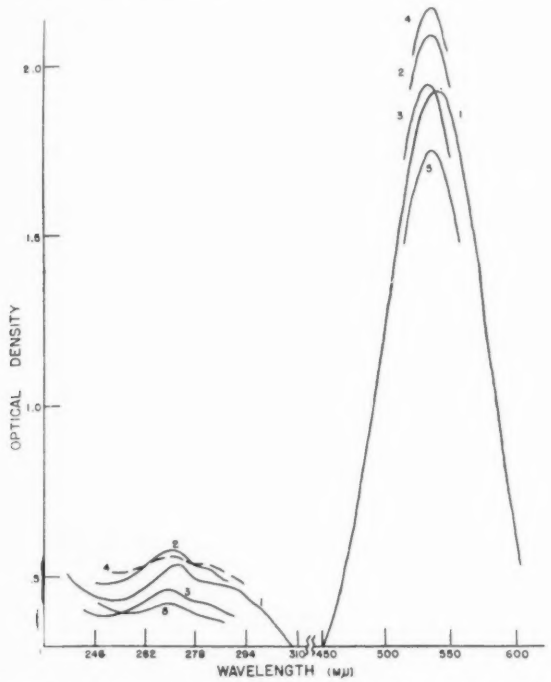


Figure 6. Spectra of equimolar betanin solutions at pH 1 to 5.

the water concentrate. This solid preparation was found to be nitrogen-free and reacted rapidly with benzidine in acid solution giving a pink compound. This same solid yellow preparation in air at 20° C slowly changed to orange (yellow plus red). It is possible that the red, nitrogen-free compound obtained by Schmidt and Schönleben *(14)* might have been this red product of the yellow pigment. Apparently closely related to this was the work of Kozlowski *(5)*, whose white and yellowish preparations yielded violet pigments on oxidation with MnO_2 in 65% H_2SO_4.

Effect of pH on absorption spectra. In many cases it appeared that the same pigment molecule appeared red or purple under different conditions. Solutions of the different pigments differed in stability. Since differences in pH were most likely responsible for these differences, the effect of pH on absorption spectra was investigated. Nine citric acid-disodium phosphate buffer solutions of varying pH were prepared and .05 ml of a concentrated solution of betanin was added to exactly 5 ml of each. The visible and ultraviolet absorption spectra were obtained immediately and are shown in Figure 6. There was practically no change in the ultraviolet but in the visible there was a change to a bluer color with increasing acidity (noticeable in the curve for pH 1). The solutions were then stored in sealed tubes for 3 months at 3° C after which time the absorption from 520 to 560 mmc was again measured. Figure 7 shows a very definite optimal pH for storage of the

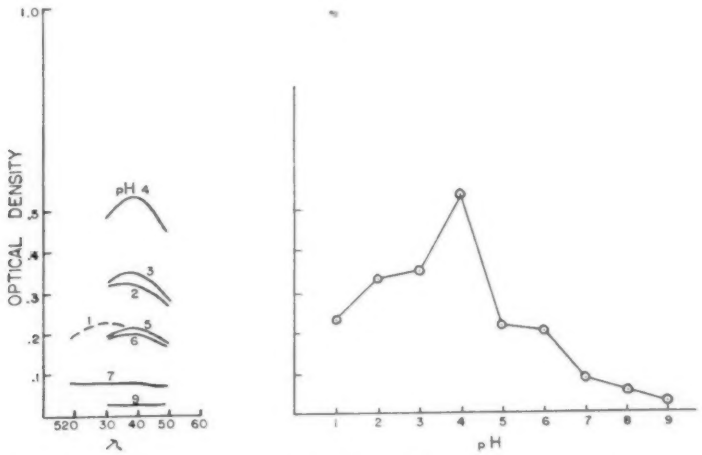


Figure 7. Optical densities of betanin solutions at pH 1 to 9 after 3 months storage at 3° C.

pigment, just below pH 4. The pigment is unstable in acid, even less stable in neutral or alkaline solutions, and most stable in solution near pH 4. Even at pH 4, 3 quarters of the pigment had decomposed during 3 months' storage in closed tubes at 3° C.

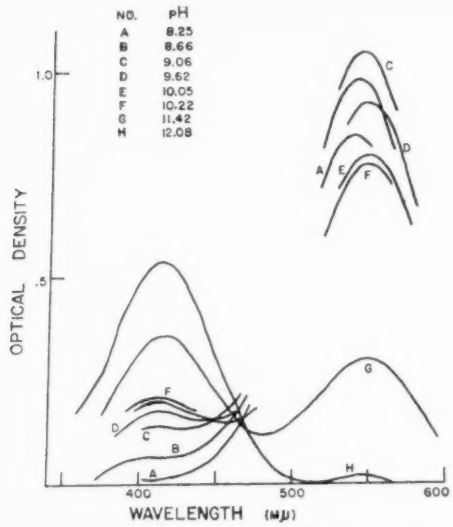


Figure 8. Betanin solutions at pH 8.25 to 12.08.

Absorption spectra of similarly prepared samples in glycine-sodium hydroxide buffer in the pH range 8 to 12 are shown in Figure 8. Assuming a neutralization reaction of:

$$\underset{\text{(purple)}}{RH^+} + OH^- \longrightarrow \underset{\text{(yellow)}}{R} + HOH$$

one can calculate the pK value for the loss of the proton by the relation

$$pH = pK + \log \frac{R}{RH^+}$$

using the extinction at the peak of the acid species as a measure of $\frac{R}{RH^+}$. Table 2 was prepared in this way from the curves in Figure 8.

TABLE 2

pH	R%	$\log \frac{R}{RH}$	pK
9.06	99.2	1.093	10.153
9.62	87.6	0.848	10.468
10.05	75.3	0.484	10.534
10.22	72.8	0.427	10.647
11.42	28.6	1.456	10.876
10.08	1.43	2.162	10.242

The average pK value calculated from these data is 10.487. A similar calculation, using the extinction of the yellow pigment at 410 mmc gives the average pK value as 10.479.

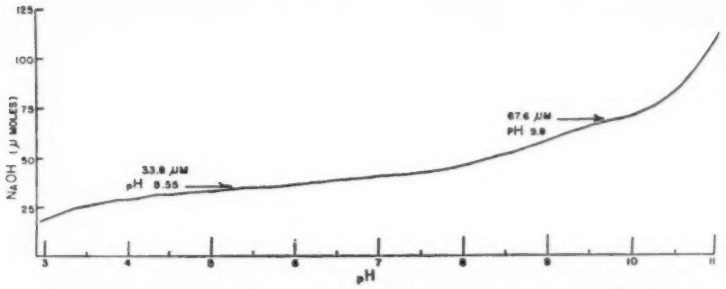


Figure 9. Microtitration of 11.89 mg of betanin with 0.1 N NaOH.

It can be seen from the curves plotted in Figure 8, that 2 changes occur in this pH range. At about pH 9.2, there is a change from red to purple as shown by the shift in wavelength of maximum absorption. Then, the purple changes to yellow at 10.5 as described above. Two isobestic points appear, one between those of purple and yellow. A similar change occurs at very low pH levels, and it appears that the pigment contains 4 ionizing groups in all, 2 carboxyl and probably 2 pyrrole immine groups.

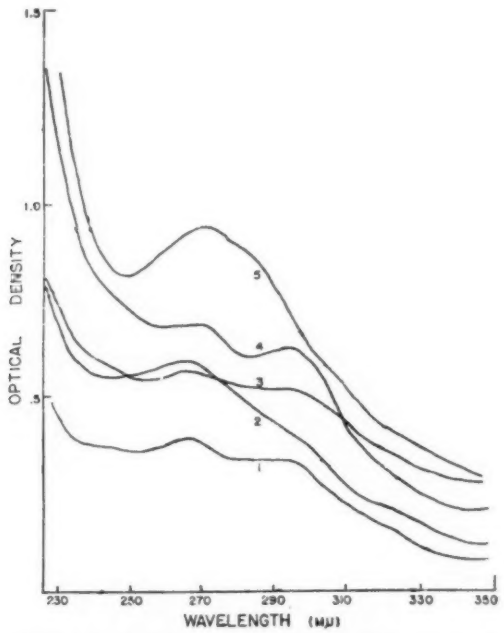


Figure 10. Ultraviolet absorption of Betanin prepared from 5 different varieties of beets. 1. Early Wonder 2. Crosby's Egyptian 3. Green Top Bunching 4. Extra Early Flat Egyptian 5. Detroit Dark Red.

Microtitration. The beet pigment was then titrated in aqueous solution with .1N sodium hydroxide in a recording micro-titration apparatus. The curve, Figure 9, does not distinguish between each of the 2 carboxyl groups or (presumed) 2 pyrrole NH groups. 11.89 mg of betanin were titrated and each inflection point required 33.8 mcM of sodium hydroxide. Therefore, 33.8 mcM = 11.89 mg and the apparent molecular weight = 352. However, two groups were titrated at each point, so the true molecular weight of betanin is very close to 704. That this figure is correct is shown by the fact that Schmidt and Schönleben *(15)* reported their crystalline potassium salt to contain about 5.5% K. Assuming one potassium atom per molecule, (the KH salt) one arrives at a molecular weight of 709. Thus, it appears that betanin has 3 nitrogen atoms per molecule instead of two, although there are probably only 2 pyrrole residues.

Varietal differences. Although different varieties of *Beta vulgaris* contain the same pigments—differing only in quantities present *(2)*, it is easier to purify betanin from certain ones. The pigment was prepared in equal concentrations from 5 different varieties by the method of Pucher, Curtis and Vickery *(8)*. A comparison of the ultraviolet absorption of betanin prepared from these is shown in Figure 10. It is interesting that *Early Wonder* was originally a selection from *Crosby's Egyptian* and *Crosby's Egyptian* was originally a selection from *Green Top Bunching*. Obviously, these G T B varieties should be used as the source of pure betanin, even though others (Detroit Dark Red) may contain more actual pigment per unit weight.

SUMMARY

The chemistry of the pigments present in the beet *(Beta vulgaris)* was investigated in some detail. Primarily from spectral evidence in the literature, it was believed that betanin is not a "nitrogenous anthocyanin" as previously supposed. A more reasonable explanation was proposed in which the pigment was assumed to be a pyrrole compound. This conclusion is supported by all the available experimental data, including spectral evidence, type reactions and breakdown products.

The pigment was titrated and a measurement of pK values and molecular weight was obtained. Reduction products of betanin were also studied. Carbon dioxide and methyl amine were identified as products of alkali fusion. A study of the yellow pigments was also made, and it was found that some were easily transformed into red compounds. At least one yellow pigment was shown to be nitrogen-free, yet could be changed into a red pigment by standing in acid solution. Thus, it is likely that some of the red pigments may be nitrogen free. It is possible that these may be furan analogues of the pyrrole pigments.

Methods of pigment preparation were reviewed briefly and it was found that the variety of *Beta vulgaris* used was important in obtaining an impurity-free betanin. Selections from Green Top Bunching were found to yield pure pigment with relative ease.

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EFFECT OF COOKING AND CARCASS PART ON THE METHIONINE AND CYSTINE CONTENT OF CHICKEN MEAT[a,b]

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In 1957 the per capita consumption of chicken and turkey meat in the United States was 25.6 pounds and 5.9 pounds, respectively *(1)*. Poultry meat has long been one of our major dietary sources for high quality protein. Studies on the amino acid content of poultry meat *(2, 4, 5, 9, 10)* are numerous.

Little information has been obtained, however, on the amino acid content of the different parts of the carcass—data of particular usefulness to dietitians. There appears, also, to be a lack of information on the effects of cooking and individual bird differences with regard to the amino acids. Research conducted on lamb, pork, and beef shows that the amino acids of these products are stable to the heat of cooking. The present study was initiated to determine (1) the cystine and methionine distribution in the white meat, dark meat, giblets and skin of poultry meat, (2) to investigate the effect of cooking, and (3) the differences in methionine and cystine content of white meat of individual birds.

PROCEDURE

General. Whole birds were baked in a covered container for 1 min per 10.5 g of weight. The time was increased to 1 min per 7 g of weight for half birds. Cooking was accomplished in a reel type oven at 325° F. Portions were dried by lyophilization. Fat was removed by a 15-hr Soxhlet extraction with petroleum ether.

One-gram samples of the moisture-free, fat-free samples were hydrolyzed in sealed tubes using 10 ml of 3 N HCl. Hydrolysis was accomplished in an autoclave at 15 lb pressure for 6 hr. The hydrolyzates were rinsed from the tube and brought to pH of 4.0 using 3 N NaOH. Darco G-60 activated charcoal (.5 g) was added to decolorize the solutions. Hydrolyzates were filtered and washed until the volume reached almost 250 ml after which the filtered hydrolyzate was brought to a pH of 6.8, made to a volume of 250 ml, and frozen in 4 oz polyethylene bottles until used for amino acid analysis.

Leuconostoc mesenteroides P-60 ATCC #8042 was the organism used for microbiological analysis. A commercial preparation of methionine or cystine media (Difco) was used. It was necessary to autoclave the cystine medium separately from the standard and test materials. They were poured together after cooling. For the standard response curve, 7 levels of the amino acid were employed, each level being replicated 3 times. Four levels of the test material were used, these also being replicated 3 times. Assay tubes were incubated at a temperature of 30° C for 72 hr. For acidimetric titration the three tubes for each level were emptied into a 150-ml. beaker *(13)* and rinsed with distilled water. The growth response curves were plotted on 1 x 2 cycles, 11 x 15 in log-log paper.

Carcass distribution. Distribution of methionine and cystine in the carcass was determined by hydrolyzing and analyzing cooked samples from composites of light meat, dark meat, gizzard, liver, heart, and skin from whole 9-week-old Hoosier White birds.

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[a] Journal Paper No. 1431 of the Purdue Agric. Exp. Station, Lafayette, Ind.

[b] From a thesis submitted to the Faculty of Purdue University by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Cooking effects. For the cooking study individual fryers were used, one half of each chicken was analyzed raw and the other half analyzed after cooking. Four hydrolyzates were made from each moisture-free, fat-free sample obtained by grinding, drying, and extracting the fat from the pectoralis major muscle of each half. Identities of the hydrolyzates from each half were maintained so that the cooked half could be compared with the uncooked portion of the same chicken.

RESULTS AND DISCUSSION

Amino acid distribution. Methionine and cystine distribution in chicken meat is shown in Table 5. It is readily evident that methionine content decreases in the order: light meat, dark meat, gizzard, heart, liver, and skin. Except for skin, low in both amino acids, cystine content decreases in exactly the order methionine increases in the various kinds of flesh sampled. This causes the total amount of the 2 amino acids to be approximately equal for all samples of carcass taken except skin.

Table 6 contains results of the analysis of variance and the multiple range test *(3)* of the methionine study. Source of the sample (light, dark, gizzard, heart, liver, or skin) was highly significant. Due to the very small error term each sample source is shown by the multiple range test to be significantly different from every other sample at the .01 level.

Analysis of variance and multiple range test for the cystine data are presented in Table 7. As with the methionine data, source of the sample has a highly significant effect on cystine content. Composite and the source x composite interaction are again non-significant. The multiple range test indicates that no individual source is significantly different from all other sources at the .01 level. Liver, however, is significantly higher than skin, and dark and light meat. At the .05 level skin is significantly lower than all the other sources, and liver is significantly higher than all samples except heart.

It is important to the dietitians to know the amount of amino acid contained in servings of all foods. For their convenience Table 8 presents the amounts of methionine and cystine in a 4 oz serving of white and dark meat, gizzard, liver, heart, skin, and white and dark meat with skin based on the protein percentage indicated. Total cystine and methionine values range from 1.50 g for white meat to .58 g for the same amount of skin. It is interesting how much a single 4 oz serving of chicken meat supplies of the suggested daily intake of 2.2 g of methionine *(11)*; also to be remembered is that cystine has a sparing action of 80–89% for methionine as a source of minimal needs of the latter *(12)*.

Cooking and individual bird variation. Methionine content and cystine content of raw and cooked poultry meat are presented in Tables 1 and 3. Statistical analysis of each appears in Tables 2 and 4.

TABLE 1

Methionine content of raw and cooked chicken meat[1]
Individual birds

	1	2	3	4	5	6	Average
Raw	2.98	2.94	2.95	2.90	2.91	2.86	2.92
Cooked	3.04	3.06	3.10	3.10	3.07	3.02	3.05
Average	3.01	3.00	3.03	3.00	2.99	2.94	

[1] Grams methionine per 16.0 g nitrogen.

TABLE 2

Analysis of variance of methionine content of raw and cooked chicken meat

Source	d.f.	M.S.	F
Bird	5	.0074	1.23
Cooking	1	.2338	38.97**
B × C	5	.0046	.77
Error	36	.0060	
Total	47		

** Significant at .01 level.

TABLE 3

Cystine content of raw and cooked chicken meat[1]
Individual birds

	1	2	3	4	5	6	Average
Raw	1.61	1.54	1.61	1.57	1.61	1.59	1.60
Cooked	1.68	1.66	1.68	1.68	1.74	1.69	1.69
Average	1.64	1.60	1.64	1.62	1.67	1.64	

[1] Grams cystine per 16.0 g nitrogen

TABLE 4

Analysis of variance of cystine content of raw and cooked chicken meat

Source	d.f.	M.S.	F
Bird	5	.0041	.60
Cooking	1	.1220	17.94**
B × C	5	.0014	.21
Error	36	.0068	
Total	47		

** Significant at .01 level.

TABLE 5

Methionine and cystine distribution in chicken meat[1]

	Light	Dark	Gizzard	Heart	Liver	Skin
Cystine	1.67	1.70	1.83	1.99	2.12	1.36
Methionine	2.97	2.90	2.82	2.75	2.42	1.71
Cystine and methionine	4.64	4.60	4.66	4.74	4.53	3.07

[1] Grams amino acid per 16.0 g nitrogen.

TABLE 6

Analysis of variance and multiple range test of methionine distribution data

Source	d.f.	M.S.	F
Source	5	1.8102	565.69**
Composite	1	.0017	0.53
(S × C)	5	.0020	0.63
Error	36	.0032	
Total	47		

** Significant at .01 level.

White	Dark	Gizzard	Heart	Liver	Skin
2.97	2.90	2.82	2.75	2.42	1.71

Any two means (grams methionine per 16.0 g nitrogen) not underscored by the same line are significantly different at the 0.1 level.

TABLE 7

Analysis of variance and multiple range test of cystine distribution data

Source	d.f.	M.S.	F
Source	5	.5639	26.85**
Composite	1	.0234	1.11
(S × C)	5	.0048	.23
Error	36	.0210	
Total	47		

** Significant at .01 level

	Liver	Heart	Gizzard	Dark	Light	Skin
	2.12	1.99	1.83	1.70	1.67	1.36
.01 level						
.05 level						

Any two means (grams cystine per 16.0 g nitrogen) not underscored by the same line are significantly different.

TABLE 8

Amino acid content of four ounce servings of chicken meat

	Total grams	Protein %	Protein grams	Meth grams	Cyst grams	Meth and Cyst grams
White	113.4	28.6	32.44	.96	.54	1.50
Dark	113.4	23.7	26.88	.78	.46	1.24
Gizzard	113.4	24.0	27.22	.76	.50	1.26
Liver	113.4	22.9	25.96	.63	.56	1.19
Heart	113.4	22.8	25.86	.72	.52	1.24
Skin	113.4	16.7	18.94	.32	.26	.58
White w/skin [1]	113.4	27.2	30.86	.90	.50	1.40
Dark w/skin [2]	113.4	22.9	26.02	.72	.44	1.16

[1] Calculated on the basis of skin as 11.6% of the edible meat.
[2] Calculated on the basis of skin as 10.9% of the edible meat.

The difference in amino acid content of individual birds did not approach significance for either methionine or cystine. When raw and cooked meat contents were averaged, the difference between highest and lowest individual bird values was only 0.09 g for methionine and 0.07 g for cystine. These data indicate that use of composite samples of birds on the same diet and of the same age is a valid assay technique.

If amino acids were destroyed during cooking one would expect a lower amino acid content after cooking than before. But both methionine and cystine, on the basis of grams of amino acid per 16.0 g of nitrogen, increased during cooking. Methionine values increased from 2.92 to 3.05 while cystine values increased from 1.60 to 1.69. These data compare favorably with those of Greenwood *et al (7)* which indicated that cooking causes methionine values of choice beef chuck to change from 2.44 to 2.49. Cystine values changed with cooking from 1.2 to 1.3.

From Tables 2 and 4 it can be seen that effect of cooking is highly significant statistically. The fact that both the sulfur-containing amino acids apparently increase during cooking poses the question as to what accounts for such an increase. Most of the fresh meat commonly consumed consists of muscle meats derived from the voluntary muscle fibers bound together with connective tissue *(6)*. The collagen content of connective tissue approaches

32% *(8)* and has the same approximate amino acid composition as gelatin, the end product of the action of heat upon collagen. Glycine represents about one-third of all the amino acids in the collagen molecule; a second third is composed of proline and hydroxyproline, the other amino acids making up the remaining third of the molecule. On the basis of grams of amino acid per 16.0 g of nitrogen, collagen contains only 0.9 g of methionine and 0.1 g of cystine. This would mean that any loss of collagen in the form of gelatin in the "drip" accumulated during cooking would change the proportion of amino acids remaining in the meat. For the half birds, averaging 551 g each, an average of 44 g of "drip" accumulated during cooking. Undoubtedly the percentages of proline, hydroxyproline, and glycine were reduced during cooking. This theory is supported by the findings of Greenwood *et al (7)* in which the proline content and glycine content of choice beef cuts were shown to decrease from 5.59 and 7.34, respectively, to 5.11 and 6.62, respectively, during cooking. The increased methionine and cystine values of cooked poultry meat can then be theorized to mean simply an increased proportion of the nitrogen present after cooking is methionine and cystine. The fact that there is an increase rather than a decrease supports the statement that cystine and methionine are stable to cooking.

SUMMARY

Microbiological assays of six hour acid hydrolyzates of chicken meat were used to determine the effects of cooking and individual bird variation on the content of methionine and cystine. The distribution of these amino acids in the various sections of the carcass was determined.

Both methionine and cystine were found to be stable to the effects of cooking by baking in a covered container. Significant increases in both amino acids occurred during cooking, apparently due to the fact that the drip lost during cooking contained very small amounts of methionine and cystine, and changed the proportion of the remaining amino acids as a percentage of the nitrogen present in the cooked meat. There were no significant differences between individual birds indicating that the use of composites is a valid assay technique.

The various segments of the chicken carcass differ significantly in their content of methionine and cystine. Light meat was highest in methionine with 2.97 grams per 16.0 grams of nitrogen. Cystine is contained in largest amounts in the liver, the level being 2.12 grams per 16.0 grams of nitrogen. Skin was lowest in both amino acids.

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